Dissecting the Pharmaceutical and Immunological Aspects of *Myrmecia pilosula* (Jack Jumper) Ant Venom Immunotherapy

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“of insects…..only the ants were troublesome…..their stings were by some esteemed not much less painful than those of a bee…”

Joseph Banks, The Endeavour Journal of Joseph Banks, August 1770

Declaration of originality

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief, no material previously published or written by another person except where indicated in the Preface, nor does the thesis contain any material that infringes copyright.

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Statement of ethical conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University. All animal experiments conducted within Chapter 6 of this thesis were approved by the Animal Welfare Committee of Flinders University and Southern Adelaide Local Health Network, Australia (Animal ethics approval number: 838/12).

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Preface

This PhD thesis combines several interconnected studies with the unifying theme of Jack Jumper ant venom allergy and its treatment modality. This thesis includes four original manuscripts that have been published in peer-reviewed international journals as described in the List of Publications table below.

These manuscripts include multiple co-authors, as the work was generated from active collaborations with other researchers within the University of Tasmania and from other institutions. The contribution of the candidate and co-authors for each manuscript is outlined in the signed a 'Statement of Co-Authorship Form'.

List of Publications

Publication title	Publication status	Publication details and Digital Object Identifier
Pilosulins: A review of the structure and mode of action of venom peptides from an Australian ant *Myrmecia pilosula*	Published	Toxicon 98 (2015) 54–61 DOI: 10.1016/j.toxicon.2015.02.013
Factors influencing the quality of *Myrmecia pilosula* (Jack Jumper) ant venom for use in in vitro and in vivo diagnoses of allergen sensitization and in allergen immunotherapy	Published	Clinical & Experimental Allergy 47 (2017) 1479–1490 DOI: 10.1111/cea.12987
Towards complete identification of allergens in Jack Jumper (*Myrmecia pilosula*) ant venom and their clinical relevance: An immunoproteomic approach	Published	Clinical & Experimental Allergy 48 (2018) 1222–1234 DOI: 10.1111/cea.13224
Pharmaceutical and preclinical evaluation of Advax adjuvant as a dose-sparing strategy for ant venom immunotherapy	Published	Journal of Pharmaceutical and Biomedical Analysis 172 (2019) 1–8 DOI: 10.1016/j.jpba.2019.04.017

Statement of co-authorship

The following people and institutions contributed to the publication of the work undertaken as part of this thesis:

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Manuscript 1. Pilosulins: A review of the structure and mode of action of venom peptides from an Australian ant Myrmecia pilosula

Located in chapter 2

Candidate was the primary author who conceived, designed and wrote the first draft of the manuscript. SGAB, NWD, NG and MDW participated in its revisions and approved the final form.

Manuscript 2. Factors influencing the quality of Myrmecia pilosula (Jack Jumper) ant venom for use in in vitro and in vivo diagnoses of allergen immunotherapy

Located in chapter 4

Candidate was the primary author who conceived, designed and executed the study. SGAB and MDW developed the Jack Jumper ant venom extraction, pooling and handling procedures. NWD and DN provided assistance with liquid chromatography experiments and relevant data analysis. HER provided general laboratory assistance. LML and HER provided general assistance with data analysis. SGAB, NWD, NG, LML, DN, HER, and MDW participated in the revisions of manuscript draft and approved the final form.

Manuscript 3. Towards complete identification of allergens in Jack Jumper (Myrmecia pilosula) ant venom and their clinical relevance: An immunoproteomic approach

Located in chapter 5

Candidate was the primary author who conceived, designed and executed the study. DG provided assistance with liquid chromatography experiments and relevant data analysis. RW provided assistance with Mass Spectrometry experiments and relevant data analysis. HER provided general laboratory assistance. SGAB, NWD, DG, NG, HER, MDW, and RW participated in the revisions of manuscript draft and approved the final form.

Manuscript 4. Pharmaceutical and preclinical evaluation of Advax adjuvant as a dose-sparing strategy for ant venom immunotherapy

Located in chapter 6

SGAB, RJH, NP, and MDW conceived the study. Candidate was the primary author who, with NP and MDW, designed the experiments. NWD provided assistance with liquid chromatography experiments and relevant data analysis. YHO and NP provided assistance with murine studies. HER provided general laboratory assistance. NP, RJW and MDW provided general assistance with data analysis. SGAB, NWD, RJH, YHO, NP, RJW, HER, and MDW participated in the revisions of manuscript draft and approved the final form.

Manuscript 5. Ant venom allergy and its clinical management (Manuscript in preparation; currently chapter 1)

We, the undersigned, endorse the above stated contribution of work undertaken for each of the above published peer-reviewed manuscripts contributing to this thesis:

Signed:

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Date:	12th December 2018	13th December 2018

Statement regarding published work contained in thesis

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Abstract

Myrmecia pilosula (Jack Jumper) is an endemic Australian ant whose sting is a frequent cause of insect allergy in parts of South-Eastern and South-Western Australia, causing severe anaphylaxis in approximately 3% of the population. The venom of *Myrmecia pilosula* ant contains IgE-binding components frequently responsible for the severe anaphylactic reactions in humans. A treatment modality based on purified *M. pilosula* ant venom extract has been developed by members of the Tasmanian Jack Jumper Allergy Program. The treatment, known as Jack Jumper Ant Venom Immunotherapy (JJA VIT), was proven to reduce the risk of severe anaphylaxis in sensitized patients and improve patients' Quality of Life. However, the current treatment is associated with frequent adverse reactions and long treatment duration. As the principal Pharmacist and Quality Manager responsible for the manufacture of JJA VIT products, it is my primary interest to continuously improve the quality, safety and efficacy of this important life-saving treatment, which is uniquely Australian.

My review of allergic reactions to the venom of stinging ants (as detailed in **Chapter 1** and **Chapter 2**) illustrated the burden and impact of *M. pilosula* venom allergy in Australia and highlights aspects of *M. pilosula* venom and JJA VIT that warrant further scientific investigations, which consequently shaped the objectives and research questions of this PhD thesis.

This research has two general objectives that were aimed to advance this treatment modality, and I have performed several interconnected studies to answer my research questions (**Chapter 3**). My first research objective was ***to improve the quality*** of the JJA VIT produced. In **Chapter 4** I explored the intrinsic and extrinsic factors that could influence batch-to-batch consistency and quality of pharmaceutical grade Jack Jumper ant venom (JJAV) extracts in the form of Active Pharmaceutical Ingredients, particularly with respect to their IgE-binding components and activities. In this analysis, I found that components of the venom with molecular weight of >20 kDa are significantly affected by elevated temperature above 40^{o}C. Notably, these venom components are capable of binding to IgE and they were of unknown identity, and their identities are revealed in **Chapter 5**. I analysed the proteome

and allergenome of JJAV separated using a combination of various gel electrophoresis and liquid chromatography techniques. To help divulge the identity of novel JJAV components capable of binding IgE, I employed a tandem Mass Spectrometry technique. From this study, I identified 17 novel JJAV proteins, including two glycoproteins, and confirmed the presence of four known Myr p and pilosulin peptides in JJAV. Most of the newly identified IgE-binding proteins were enzymes, including phospholipase A_2, hyaluronidase, arginine kinase, and dipeptidyl peptidase IV.

My second research objective was ***to improve the safety and efficacy*** of JJA VIT. For this purpose, I analysed the response of subjects undergoing JJA VIT with respect to their IgE-binding recognition to JJAV components pre-treatment and I correlated this information with treatment tolerability and efficacy. I subsequently linked this clinical data with the various JJAV components identified *via* tandem Mass Spectrometry and report my results in **Chapter 5**. In this study, I established correlations between recognition of certain IgE-binding bands with JJAV-specific IgE titre by ImmunoCAP, intradermal test threshold, and treatment-related issues. Finally, driven by the relative difficulty in obtaining pharmaceutical grade JJAV extracts and the recent increase in demand to treat patients with JJAV allergy within Tasmania and interstate, I explored the safety and efficacy of treatment with low-dose JJA VIT using Advax™ adjuvant. In order to enable a clinical trial using this novel combination product, I performed fundamental pharmaceutical and immunological studies. In **Chapter 6** I report the physicochemical and microbiological stability and murine immunogenicity of low-dose JJA VIT in combination with Advax adjuvant. I observed that JJA VIT formulated with Advax is both physicochemically and microbiologically stable for at least 2 days when stored at 4 and 25^{o}C, with a trend for an increase in allergenic potency observed beyond 2 days of storage. Importantly, JJA VIT formulated with Advax significantly increased the production of JJAV-specific IgG, consistent with a JJAV antigen-sparing effect of the adjuvant, which supports the use of Advax adjuvant with JJA VIT in future clinical trials.

Overall, my PhD project has advanced our knowledge on the pharmaceutical and immunological aspects of JJA VIT and provides a robust platform to enhance the quality, safety and efficacy of this life-saving treatment modality.

Part I: INTRODUCTION

Chapter 1: Systemic anaphylactic reactions to the venoms of stinging ants and its management

1. Overview

Hymenoptera venom allergy is characterised by systemic anaphylactic reactions that occur in response to stings from members of the Hymenoptera order (1). Stinging by social Hymenoptera such as ants, honeybees and vespids, is one of the 3 major causes of anaphylaxis along with food and drug exposure (2, 3), and it accounts for up to 43% of anaphylaxis cases and 20% of anaphylaxis-related fatalities (4, 5). The prevalence of systemic anaphylactic reactions to Hymenoptera stings, predominantly by honeybees and *Vespula* species, ranges from 0.5% to 7.5% in adult and up to 3.4% in children (6-8). Despite their recognition as being of considerable public health significance (9), stinging ant venoms are relatively unexplored in comparison to other animal venoms and may be overlooked as a cause of venom allergy (10, 11). Indeed, the venoms of stinging ants may be the most common cause of anaphylaxis in ant endemic areas (12-15). A better understanding of the natural history of venom allergy caused by stinging ants, their venom components, and the management of ant venom allergy is therefore required. The following sections will provide a global view on allergic reactions to the venoms of stinging ants and the contemporary approach to diagnose and manage ant venom allergy, with emphasis on the Australian context.

2. Epidemiology of ant venom allergy

Ants (order Hymenoptera, family Formicidae) are very biodiverse organisms with 21 recognized subfamilies and more than 13,000 described species (16, 17). Sixteen subfamilies of ants and 1900 living species have a stinging apparatus and use injected venom for defence against predators, prey capture, and social communication (18, 19). However, most stinging ants are not aggressive and rarely sting large organisms. There are about nineteen genera of ants that have been associated with venom allergy in humans, but only members

of four genera (Figure 1), namely *Solenopsis spp*., *Myrmecia spp*., *Pachycondyla spp.*, and *Pogonomyrmex spp.* have caused anaphylactic reactions and fatalities frequently enough to be considered important medical problems. The risk of envenomation and anaphylactic reaction by a particular species of stinging ant depends on complex interactions between the likelihood of human contact, species aggressiveness, efficiency of the venom delivery apparatus and venom allergenicity (20, 21).

2.1. Causative species

2.1.1. *Solenopsis spp*.

The genus *Solenopsis* comprises small to medium sized ants of 2 to 6 mm in length. They are commonly referred to as fire ants due to the fierce burning pain inflicted by their sting (22). There are over 60 known species of fire ants within this genus (23), but only five species have been associated with sting allergy. Fire ants are usually found in large colonies of 250,000 ants or more, and up to 600 mounds per acre may be found in heavily infested areas (22). Fire ants use pheromones to recruit large numbers of worker ants to attack intruders *en masse* (24), thus achieving a significant antigenic load despite their small individual stings. The medically important species of fire ants are *S. richteri*, *S. invicta*, *S. aurea*, *S. geminata*, and *S. xyloni*.

S. richteri, the black fire ant, was introduced into the United States of America (USA) from Uruguay or Argentina by cargo vessels through the port of Mobile, Alabama, in 1918 (25), while *S. invicta*, the red fire ant, was introduced to the USA from Brazil around the time of World War II (26). They are often referred to as Imported Fire Ant (IFA) to distinguish them from the native North American fire ant species, *S. aurea* (desert fire ant)*, S. geminata* (tropical fire ant), and *S. xyloni* (southern fire ant). The red IFA, *S. invicta* has also been established in Asia and Australia, while the black IFA, *S. richteri* has been carried as far as Saudi Arabia (24, 27-31). The tropical fire ant, *S. geminata*, commonly found in Mexico, Central America and the Caribbean islands (32), has become a serious pest and the cause of ant sting allergy in Southeast Asia, United Arab Emirates, and on some Pacific islands including Okinawa and Guam (33-35).

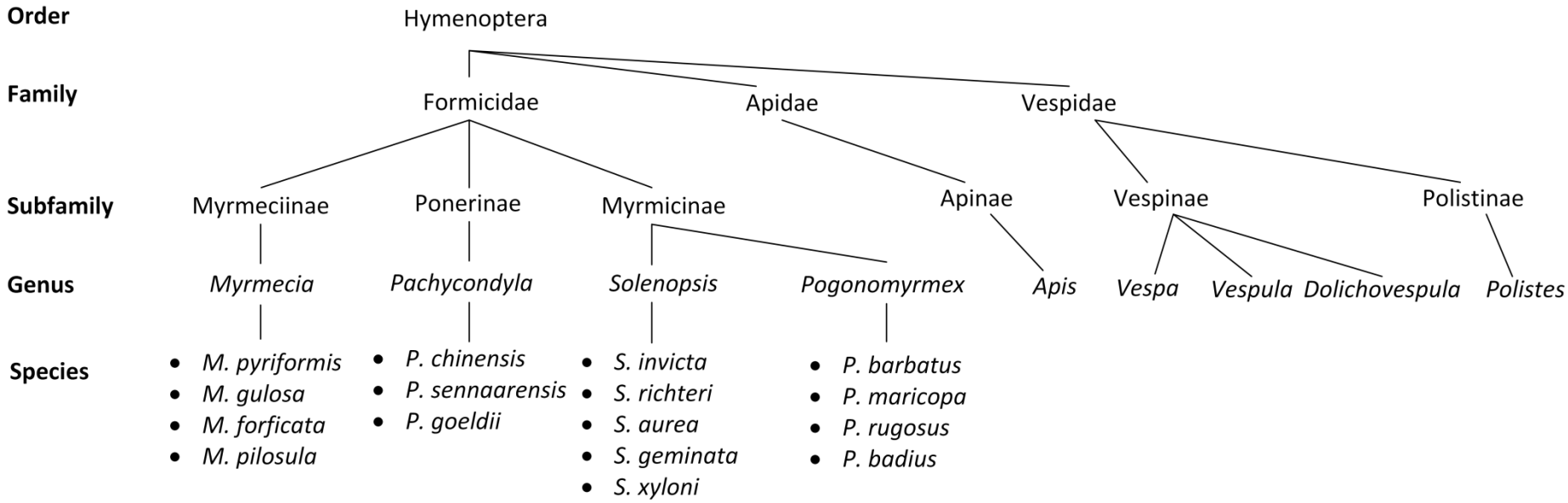

Figure 1. Schematic diagram depicting phylogenetic relationships of social Hymenoptera, with an emphasis on stinging ants frequently linked to anaphylactic reactions and death in humans

2.1.2. *Myrmecia spp*.

The genus *Myrmecia* is one of the most primitive subfamilies of ants, and is widely distributed in and virtually endemic to Australia, with only one species found in New Caledonia (36, 37). A species of *Myrmecia* ants, *M. gulosa*, was among the earliest Australian insects to be discovered by Joseph Banks and Daniel Solander at Botany Bay in 1770 when they were on shore from Captain Cook's "Endeavour" (21). *Myrmecia spp*. are highly aggressive ants (38, 39), characterised by the prominent pair of powerful mandibles and large eyes that enable them to use visual cues to navigate (40). There are 89 recognized species within this genus, which can be broadly divided into two groups based on their morphological characteristics (36, 41). The first group comprises ants commonly known as "bulldog ants", "bull ants", "bulljoe ants", "giant bull ants", or "inchman ants" (Figure 2). Species in this group all share a large body length between 20 to 30 mm (41). The typical species in this group is exemplified by the "bulldog ant", *M. pyriformis*, which is brown in colour and is common to open sclerophyll woodland in South-Eastern Australia (42), *M. gulosa*, a slender ant with long legs and a variety of colours (41, 43), and the "inchman ant", *M. forficata*, with a purple-brown body and black abdomen that predominates in Tasmania (41).

The second group includes smaller-sized ants, typically 10 to 12 mm in length, which are commonly known as jumper ants (Figure 2). The most notable species and an archetype for this group is *M. pilosula*, an exceptionally aggressive stinging ant with highly developed vision that is attracted to movement (20, 40). *M. pilosula* has a jet black body and yellow or orange mandibles and leg tips. These ants move in short jerks and jumps, leading to the names "jumper ant", "hopper ant", "jumping jack", "black jumper", "jackie jumper", and "jack jumper" (11, 44). *M. pilosula* is a "species complex", comprised of several closely related sibling species with subtle morphological differences (45-47). Components of the species complex have been recently reviewed, illustrated and keyed; the six known members of the complex are *M. banksi* sp. nov., *M. croslandi* Taylor 1991, *M. haskinsorum* sp. nov., *M. imaii* sp. nov., *M. imparternata* sp. nov, and *M. pilosula* Fr. Smith 1858 (44). *M. pilosula* form small colonies of 100 to 1000 ants and their nests range from a single hole to mounds up to a meter in diameter with multiple entrances (Figure 2), typically surrounded

by a scattering of fine gravel (11, 20, 48). *M. pilosula* is found in sandy soiled areas starting from north of Brisbane through to the higher altitude and rural areas of South-Eastern Australia and west to the vicinity of Denmark in Western Australia (42, 43, 49). Several ant species from this genus are an important cause of anaphylactic sting reactions in Australia (21, 41). Allergy is common, particularly to the venom of *M. pilosula* in Tasmania, regional Victoria, the Adelaide Hills and southern NSW (11, 21, 48, 50). Unlike the massive ant attacks typical of IFA, single or few stings from *M. pilosula* are sufficient to cause anaphylactic reactions in humans (14).

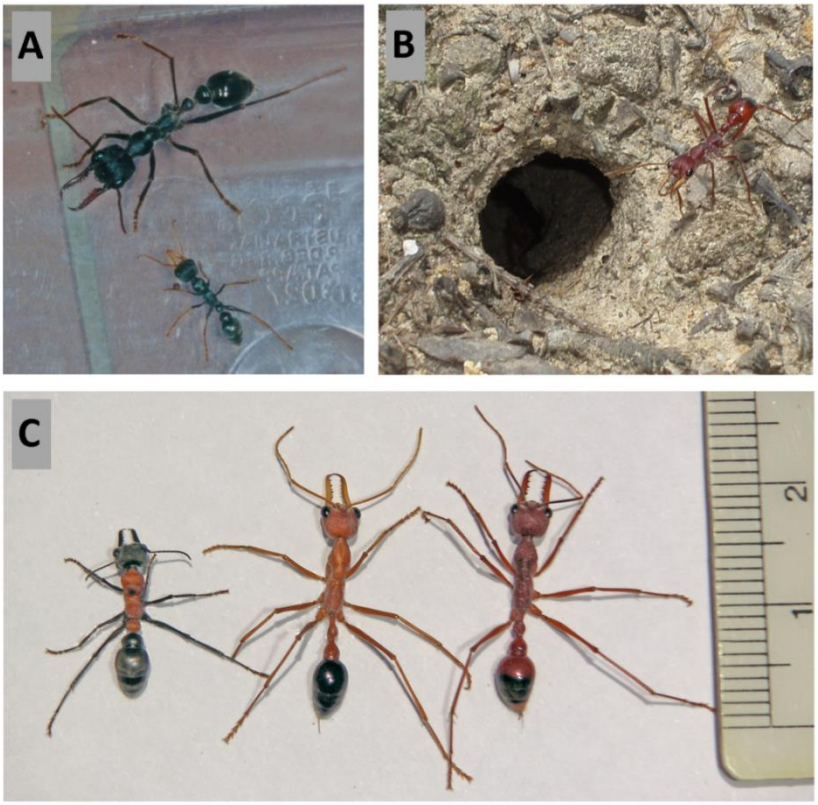

Figure 2. Pictures of some *Myrmecia* ant species

Panel A: large bulldog ant, *Myrmecia pyriformis*, and a smaller jack jumper ant, *Myrmecia pilosula*. Panel B: A *Myrmecia* ants' nest showing a single entrance. Panel C: A jumper ant, *Myrmecia nigrocincta* and bulldog ants of the *Myrmecia gulosa* species group. Photographs are courtesy of Professor Simon Brown.

2.1.3. *Pachycondyla spp*.

The ants of the genus *Pachycondyla* [now reclassified as *Neoponera spp.* (51)] originate from the neotropics, but have been spread to numerous locations by commerce, including to the South-Eastern USA (52-54). The genus comprises approximately 200 species, most of which appear to be predators, which subdue their prey with their venom (55). Two species, *P. chinensis* and *P. sennaarensis*, have been recurrently reported to cause anaphylactic sting reactions in humans (56-61), while another species, *P. goeldii,* has been associated with ant sting anaphylaxis in a single case report (62).

P. chinensis (Chinese or Asian needle ant) originates from Far East Asia, but is now widely distributed throughout Asian-Pacific countries (53, 63). *P. chinensis* are 4 to 6 mm in length, with a shiny brownish-black body, orange-brown legs and a well-defined stinger (52). Similar to *M. pilosula*, *P. chinensis* is also a species complex (64). *P. chinensis* colonies are composed of a few hundred workers, and nests tend to be found in damp areas near mature forest, within rotten wood, or in old wooden houses (60, 65). Although not an aggressive species, *P. chinensis* is known to have a sting that results in sharp, low-pain intensity that persists for 30 minutes or more (23, 65-67).

P. sennaarensis (samsum or sword ant) has a black and relatively slender 4 to 6 mm long body and is widely distributed throughout the African tropics, Arabian peninsula and Southern Iran (54, 66, 68). Colonies of *P. sennaarensis* typically contain several thousand workers in underground nests approximately one meter deep (55, 69). *P. sennaarensis* requires an environment with high humidity, so they are commonly found around human settlements with a strong presence in gardens and buildings. *P. sennaarensis* is an “opportunist” ant; in its natural habitat it has a mainly granivorous diet during the dry season and feeds on animal prey during the rainy season, but in urban areas the ants scavenge on human food refuse (54, 66, 70). *P. sennaarensis* has a reputation for its aggressiveness and extremely painful sting, with pain that can persist for up to 4 hours (34, 55, 68, 71).

2.1.4. *Pogonomyrmex spp.*

Certain members of the genus *Pogonomyrmex* have been reported to cause ant sting anaphylaxis in the USA. Commonly referred to as the harvester ant, they have large mandibles to collect and feed on seeds from their native habitat (72, 73). They are 5 to 9 mm in length and are reddish to blackish in colour (74). Harvester ants usually construct their nests in the sand or soil, and may be more than 6 meters deep, containing large colonies of more than 20,000 ants (75). The genus comprises over seventy species and are found in arid grasslands and deserts of North and South America (76). Three species, *P. barbatus* (red harvester ant), *P. maricopa* (Maricopa harvester ant), and *P. rugosus* (rough harvester ant) have been reported to cause anaphylactic sting reactions in humans, particularly around the South-Western North American desert (72, 73, 77). Although less aggressive than members of the genus *Solenopsis*, harvester ants also sting *en masse* and the sting of these ants has long been recognized for their extreme painfulness that may last for up to 24 hours (23, 74, 76, 78). The pain has been described as “ripping muscles or tendons” and like “turning a screw in the flesh” (18). The venom causes localized sweating and piloerection at the sting site and is accompanied by pain and tenderness in nearby lymph nodes (79). It is these properties that have earned them the reputation of being dangerous adversaries to humans (9).

2.1.5. Other ant species associated with allergic reactions

In addition to the four genera of stinging ants of medical importance described above, at least 16 other species of ants have also been associated with allergic reactions in humans (Table 1). One of these ants, *Rhytidoponera metallica*, is a frequent cause of ant sting anaphylaxis in Queensland (41, 80-82), with some cases have also been reported from country New South Wales (41, 83). *R. metallica* is a native Australian ant species of the subfamily Ectatomminae (63). It is an omnivorous diurnal predator, which feeds on insects and invertebrates (84), and forager of seeds and honeydew (85). The worker ant is 5 to 7 mm long with variation in a metallic colour of green, purple, red and violet (85). *R. metallica* is widely distributed throughout Australia (42). It mainly inhabits open and moderately wooded areas, including metropolitan parks and gardens (85).

Table 1. Other ant species reported to cause allergic reactions in humans

Species	Common name	Geographic location of incident	Number of incident(s)	Remarks	References
Dinoponera gigantea	False tocandira ant	Bonito town, Brazil	1	Patient recovered	(86)
Hypoponera punctatissima		Storrington, USA	1	Patient recovered	(63, 87)
		Roosevelt Island, USA	1	Patient recovered	(63)
Odontoponera denticulata		Thailand	Not specified		(88)
Odontomachus bauri	Trap-jaw ant	Caracas, Venezuela	1	Patient recovered	(89)
Odontomachus simillimus	Dalakadiya	Kurunegala, Sri Lanka	1	Patient died	(90)
Camponotus compressus	Common Indian black ant; Kulpe	Karnataka, India	1	Patient recovered	(91)
Cataglyphis bicolor	Sahara desert ant	Benha District, Egypt	Not specified		(92)
Cerapachys spp.	Cannibal ant	Australia	Not specified		(24)
Crematogaster matsumurai vagala		Iksan, Korea	1	Patient recovered	(93)
Formica rufa	Wood ant	Central Europe	1	Patient recovered	(63, 94)
Oecophylla smaragdina	Weaver ant; green tree ant	Northern Australia	Not specified		(95)
Pseudomyrmex ejectus	Twig ant; oak ant	Georgia, USA	1	Patient recovered	(63, 87)
	Twig ant; oak ant	Florida, USA	1	Patient recovered	(63)
Rhytidoponera metallica	Greenhead ant; pony ant	Queensland, Australia	47	Cases spanning 17-year period	(41, 63, 80, 81, 87, 96)
Tetraponera rufonigra	Bi-coloured arboreal ant	Bangkok, Thailand	1	Patient recovered	(88, 97)
	Hathpolaya	Kurunegala, Sri Lanka	1	Patient recovered	(90)
Tetramorium caespitum	Pavement ant	South Carolina, USA	1	Patient recovered	(63, 87, 98)
Heteroponera carinifrons		Chile	Not specified		(99)

2.2. Incidence and prevalence of ant sting allergy

2.2.1. *Solenopsis spp*.

Stings by the IFA are a major cause of systemic allergic reactions in South and Central America as well as in South-Eastern USA (33, 100). Isolated cases of anaphylactic sting reactions due to IFA have also been reported in Australia, Canada, Saudi Arabia, Spain and Switzerland (24, 31, 101-103). In heavily infested areas in Southern USA, sting attack rates range from 13% to 58% of the population per year with the highest attack rates occurring between April and September (22, 26, 104). In these endemic areas, the incidence of systemic anaphylaxis due to IFA stings exceeds that of other species of Hymenoptera (9, 12). Rhoades *et al*. estimated nearly four new cases of IFA envenomation per 100,000 population per year (105). In one survey of 29,205 physicians, it was reported that 20,755 patients per year were treated for IFA sting reactions, of whom 413 (2%) had life-threatening anaphylaxis (106). Other surveys reported that 0.6% to 16% of those stung by IFA developed anaphylaxis (107). Over 80 fatal and two near-fatal anaphylactic reactions caused by IFA stings have been reported (107-109). While the overwhelming majority of allergic sting reactions to fire ants are caused by IFA, cases of systemic allergic reactions caused by the less common species of fire ants, *S. aurea*, *S. geminata* and *S. xyloni* have also been described in India, Indonesia, Thailand and USA (32, 35, 63, 77, 110-113).

2.2.2. *Myrmecia spp*.

Myrmecia spp. is the predominant cause of ant sting allergy in Australia, implicated in 89% anaphylaxis cases due to ant stings (41). The prevalence of systemic allergic reaction to the venom of *Myrmecia* ants is 0.8% in Queensland, 2.4% in regional Victoria and 2.7% in Tasmania (13, 50, 81). Nationally, 60.8% of the 347 hospitalised cases involving ant sting allergy between 2002 and 2005 have been attributed to *Myrmecia spp.* (114). The jumper ant *M. pilosula* is the principal cause, accounting for 57% of all of ant sting anaphylaxis incidents in one large nationwide study (41). The largest number of stings by *M. pilosula* resulting in anaphylaxis involved residents of Victoria (37.5%) and Tasmania (26.5%) (114). In Western Australia the bulldog ants, *M. gratiosa* and *M. nigriscapa*, and a jumper ant, *M. ludlowi*, have been responsible for 10 cases of ant sting anaphylaxis (82). In Tasmania,

where 12% of the population is exposed to *M. pilosula* stings every year (13), it accounts for 15.4% of the total allergic reactions and cases of anaphylaxis, and between 21% to 25% of anaphylaxis cases treated with adrenaline at the Emergency Department of the Royal Hobart Hospital, the only public teaching hospital in Southern Tasmania (11, 115). Around 1% of the population in Tasmania is at risk of severe and potentially life-threatening anaphylactic reactions to *M. pilosula* stings (13).

Fatal anaphylaxis from *Myrmecia spp*. stings has also been documented. Three ant-sting related fatalities were recorded in NSW in 1931 and one fatality was recorded from Tasmania in 1963 (116, 117). Additionally, six documented cases of ant sting-related fatalities were attributed to *M. pilosula, M. pyriformis*, or *M. forficata* in the Australian Coronial records between 1980 and 1999; all the deceased were living in either NSW or Southern Tasmania (11, 14). Examination of the National Mortality Database maintained by the Australian Institute of Health and Welfare between January 1997 and December 2005 shows 20 cases of Hymenoptera sting-induced anaphylaxis fatalities, with 30% of the fatalities attributed to *Myrmecia spp.* and arthropods other than honeybees and vespids (118). In another study, using data extracted from the Australian National Coronial Information System database, a total of 34 deaths due to Hymenoptera stings or tick bites were recorded between January 2000 to December 2013, of which 6% (n=2) were caused by ant-sting related anaphylaxis (119, 120). Taken together, the incidence of fatalities due to ant sting anaphylaxis in Australia occurred at a rate of approximately 0.01 per million populations per year.

2.2.3. *Pacycondyla spp*.

Numerous incidences of allergic sting reactions to the venom of *P. chinensis* have been reported, mostly from South Korea, but some cases from Japan and South-Eastern USA have also been reported (53, 56, 57, 60, 121-126). There have been multiple reports of allergic sting reactions caused by *P. sennaarensis* in Saudi Arabia, South-Eastern Iran and the United Arab Emirates (UAE), including five cases of fatal sting anaphylactic reactions in the UAE (34, 58, 59, 61, 68, 127-131). A single case of ant sting anaphylaxis caused by *P. goeldii* was recently reported in Brazil (62). The proportion of the population that experienced systemic

allergic reactions and life-threatening anaphylactic reactions to *P. chinensis* stings in an ant-endemic area of South Korea have been reported to be 2.1% and 1.2%, respectively (122), which is comparable to those seen in the endemic areas for IFA and *Myrmecia spp.* in USA and Australia respectively.

2.2.4. *Pogonomyrmex spp*.

Stings from ants of the genus *Pogonomyrmex* occasionally result in allergic reactions. At least two deaths and a single anaphylactic reaction were attributed to stings of the red harvester ant, *P. barbatus*, between 1950 and 1974 (77, 132, 133). During this period, harvester ant stings were also implicated in severe allergic reactions in at least 27 people in Texas (134). Further cases of anaphylactic reactions in four patients and large local reactions in another four patients caused by either *P. maricopa* or *P. rugosus* were reported in Arizona in 1977 (73). More recently, three cases of anaphylactic reactions by *P. barbatus*, *P. maricopa* and *P. rugosus* were reported from Arizona and Texas in the early 2000s (63, 113, 135). The Florida harvester ant, *P. badius*, has a venom that appears capable of causing allergic reactions (see section 3 below), but anaphylactic reactions to this species have not been reported (136).

3. Ant venom components, venom allergens and their immunological cross-reactivity potential

Knowledge of the composition of ant venoms, particularly venom allergens and their cross-reactivity potential is a prerequisite for the accurate diagnosis and treatment of ant venom allergy. However, despite the rapid progress in the fields of genomics, transcriptomics and proteomics over the last decades, venom allergens of stinging ants were seldom comprehensively analysed. The limited amount of venom present in the venom sac of ants hinders its analysis and consequently limits the available data on ant venom chemistry and composition (137, 138). The currently available information shows that ant venoms include a complex mixture of chemicals including proteins, peptides,

biogenic amines, hydrocarbons and other organic molecules (9, 18, 137-139). However, only those venoms that contain proteins and large peptides cause allergic reactions (140).

Compared to other Hymenoptera venom allergens, only a limited number of stinging ant venom allergens have been characterized and named according to the International Union of Immunological Societies (IUIS) criteria for allergen nomenclature (141). Under this system, allergens are designated according to the accepted Linnéan taxonomic name of their source as follows: the first three letters of the genus, space, the first letter of the species, space, and followed by an Arabic numeral, which is assigned to individual allergens in the order of their identification. Where isoallergens and variants of an allergen group have been identified, they are designated by suffixes of a period followed by four Arabic numerals. The first two numerals (01 to 99) refer to a particular isoallergen, and the two subsequent numerals (01 to 99) refer to a particular variant of a particular isoallergen (141). Additionally, allergens may also be referred to as major and minor depending on whether in more or less than 50% of allergic patients the corresponding allergen specific IgE can be detected (142).

3.1. *Solenopsis spp*.

The venom of IFA is composed of a 90 to 95% non-aqueous phase containing non-allergenic piperidine alkaloids named Solenopsins, primarily of the trans stereoisomer of 2,6-dialkylpiperidines (143-147). These alkaloids are responsible for local sting reactions and the development of a characteristic sterile pustule formation at the sting site (148). The aqueous phase of *S. invicta* venom contains approximately 10–100 ng of protein per sting (149). Forty-six proteins with molecular weights of <10 kDa to 44 kDa have recently been identified in the venom of *S. invicta*, 21 of which are toxins. These proteins include various neurotoxins, myotoxin 2-like proteins, and ponericin-like peptides (150). Immunoblot studies identified five IgE-binding bands in *S. invicta* venom, two of which are major allergens (151). Four distinct allergenic proteins, designated Sol i 1–4, have been isolated, sequenced and characterized (149, 152).

Sol i 1 is a major allergen in IFA venom, but comprises only 2 to 4% of the total venom protein (149, 153). It is a 37 kDa phospholipase A_1 protein, with 35% sequence homology to those found in vespid venom (19, 154, 155). It contains *N*-linked carbohydrates, but a study using recombinant carbohydrate-free Sol i 1 indicated that IgE reactivity appears to be against protein determinants (156). Some honeybee and vespid venom-sensitized individuals produce IgE antibodies that cross-react with Sol i 1 (157), and as such these individuals may experience allergic reactions to their first IFA sting (140).

Sol i 3 is the second major allergen in IFA venom and makes up about 15 to 25% of the venom protein (33). It has a molecular weight of 24 kDa and is a member of the antigen 5 family of vespid venom (27, 153). Sol i 3 does not contain *N*-linked carbohydrates and displays 47% sequence homology to antigen 5 from vespid venom (Ves v 5) (152). However, the three-dimensional structure of Sol i 3 has been determined (158), and shows very little surface conservation relative to antigen 5 (27), which is consistent with a lack of immunological cross-reactivity (140).

Sol i 2 displays the highest protein concentration in IFA venom, representing 67% of the total (22). It is a disulfide-linked homodimeric protein of 28 kDa (72, 159). The three-dimensional structure of Sol i 2 suggests that it is related to pheromone-binding proteins (160). Sol i 4 is a monomeric protein of 13.3 kDa (100), and a member of the Sol i 2 family. Sol i 4 represents about 9% of the IFA venom protein and is the most basic protein component in IFA venom with an isoelectric point (*pI*) of 10.08 (107). It has about 35% sequence homology and one less cysteine residue than Sol i 2 monomers, which prevents dimerization (72, 140, 149, 159). Sol i 2 and Sol i 4 are minor allergens and neither contain *N*-linked carbohydrates (27, 153).

The Sol 2 antigen group shows a lower degree of sequence conservation among different fire ant species, while the Sol 1 and Sol 3 antigen groups appear to be more conserved (27, 154, 161). However, studies on sera from patients allergic to the native fire ant species *S. aurea* and *S. xyloni* show some evidence of immunological cross-reactivity with the Sol i 2 and Sol r 2, suggesting that similar IgE-binding epitope(s) is/are present in the venom of these species (32, 162). *S. richteri* venom does not contain a protein

analogous to Sol i 4, but a homologous allergen Sol g 4 is present in the venom of *S. geminata* (27).

3.2. *Myrmecia spp*.

Studies on the venom components of *Myrmecia spp*. have been performed on *M. pilosula, M. gulosa* and *M. pyriformis.* The currently known composition, structure and function of the venoms, with a particular emphasis on the Pilosulin peptides from *M. pilosula* venom, have been subjected to an in-depth review (163) (see following chapter for details of the review). The venoms of *M. pilosula* and *M. pyriformis* contain phospholipase A_2, phospholipase B, hyaluronidase, acid and alkaline phosphatases, histamine-releasing activities, and a heat-sensitive haemolytic factor (164-168). Analysis of *M. gulosa* venom shows the presence of 13 unique peptides named MIITX-Mg and six proteins, including a venom antigen 5, a venom dipeptidyl peptidase IV, a phosphatase, an esterase, and a hyaluronidase (169-171). Histamine has been found in all three *Myrmecia* venoms, with *M. pyriformis* venom containing significantly more than honeybee venom (166, 167, 170), and *M. forficata* venom is also exceedingly rich in histamine (172). Pharmacological studies on *M. pilosula* and *M. pyriformis* venoms showed that they are able to promote the synthesis of arachidonic acid metabolites (167), which is most likely related to the presence of phospholipase A_2 in the venoms (173). In addition to their allergenic potentials, the venoms of *Myrmecia* ants are also highly toxic to mammals (23), with intraperitoneal administration of *M. pyriformis* venom in mice inducing agitation and aggressiveness, which is usually followed by a quiescent period before death (166). The lethal dose for 50 percent of the animals (LD_{50}) for *Myrmecia* ant venoms compared to honeybee (*Apis mellifera*) venom in mice is shown in Table 2 below.

Three peptides in *M. pilosula* venom have been recognized as allergens by the International Union of Immunology Societies, and are named Myr p 1, Myr p 2 and Myr p 3 (174), and another peptide in venom is a histamine-releasing peptide Pilosulin 5 (175). Myr p 1 is a monomer peptide of 6067 Da (also called Pilosulin 1 and M-myrmeciitoxin-Mp1a); Myr p 2 is a disulfide linked heterodimer of 5608 Da (also called Pilosulin 3 and delta-Myrtoxin-Mp1a); Myr p 3 is a disulfide linked homodimer of 8198 Da (also called Pilosulin 4);

and Pilosulin 5 is a disulfide linked homodimer of 8546 Da (175-179). All four peptides are highly basic and multiple isoforms of these peptides have been identified by genetic analysis or mass spectrometry (163). Myr p 2 is the only major allergen in *M. pilosula* venom and is identified by 78% of the population with *M. pilosula* venom allergy (174). The three dimensional structures of Myr p 1 and Myr p 2 have been determined by NMR spectroscopy (138, 178). A number of uncharacterized components of >20 kDa with IgE-binding capacity and <10kDa without IgE-binding capacity are known to be present in the *M. pilosula* venom (174).

Table 2. Lethal dose of various stinging ant venoms in animals

Species	LD_{50} (mg/kg)[a]	Reference
Myrmecia spp.		
M. pilosula	5.7	(23)
M. pyriformis	2 – 10	(166)
M. nigriceps	7.3	(18)
Pachycondyla spp.		
P. senaarensis	420.7	(180)
P. goeldii	160	(180)
Pogonomyrmex spp.		
P. maricopa	0.15	(18)
P. badius	0.42	(78)
P. rugosus	0.47	(74)
P. barbatus	1.29	(9)
Dinoponera spp.		
D. gigantea	38	(181)
Apis spp.		
A. mellifera	3.5	(23)

[a]Venoms were administered to mice except for *P. barbatus* where it was administered to dogs, and to crickets for *Pachycondyla spp*.

The cross-reactivity potentials of *Mymercia* ant venoms have been studied. Using 32 sera from subjects allergic to the stings of *Myrmecia* ants and venom extracts from six *Myrmecia* ants species (*M. pilosula*, *M. pyriformis*, *M. gulosa*, *M. nigrocincta*, *M. tarsata*, and *M. similima*), it was shown that some degree of cross-reactivity exists between the different *Myrmecia* ant venoms analysed (182). Another study using sera from patients with IFA venom and *Myrmecia* venom allergies, showed that IFA and *Myrmecia* venoms have a

very low degree of cross-reactivity (161). Except for the Pilosulin peptides, IgE-binding components in other *Myrmecia* ant venoms have not been characterized thus far.

3.3. *Pachycondyla spp*.

Preliminary evidence indicated that the venom of *P. chinensis* likely contains hyaluronidase, phospholipase A_2, histamine, formic acid and terpenes (121). One-dimensional polyacrylamide gel electrophoresis (PAGE) analysis of *P. chinensis* venom extract showed the presence of at least 10 protein bands ranging from <14 to 90 kDa, with an intense band at approximately 29 kDa (57, 183). Immunoblot analysis showed the presence of two IgE-binding bands with an apparent molecular weight of 27 and 29 kDa (57). The presence of these IgE-binding bands was confirmed in a separate study, which also identified six additional IgE-binding bands with apparent molecular weights of 12, 22, 25, 31, 46, and 58 kDa (56). Results from ELISA inhibition studies failed to detect cross-reactivity between *P. chinensis* and IFA venom, but demonstrated some cross-reactivity with yellow jacket venom and a very high degree of cross-reactivity with *P. solitaria* venom (56, 57, 122).

Analysis of *P. sennaarensis* venom showed the occurrence of a volatile compound phenol-2,4-bis(1,1 dimethylethyl), a few terpenes including an alarm pheromone trimethyl pyrazine, and a small amount of unidentified peptides (55, 69, 180). Comparative toxicological analysis of 12 *Pachycondyla spp.* ant venoms, which include *P. sennaarensis*, confirmed their paralysing and lethal effects on animal prey (180). The LD_{50} of *Pachycondyla spp*. venoms are, however, much higher compared to the other medically important stinging ant venoms (Table 2).

An immunoblot study of *P. sennaarensis* venom identified five IgE-binding proteins, two of which had an apparent molecular weight of 16 and 24 kDa (68, 127). A cross-reactivity study using immunoblot and sera from patients sensitized to *P. sennaarensis* venom showed some cross-reactivity with IFA venom. All sera tested bound to the 24 kDa allergen Sol i 3 and some of the sera also bound the 13 kDa allergen Sol i 4 (127, 140). Further characterization of the 24 kDa IgE-binding band in *P. sennaarensis* venom was completed using *P. chinensis* venom, which resulted in the identification of a 23 kDa allergen named

Pac c 3 (183). Pac c 3 is a member of the antigen 5 family of proteins and has 54% homology to Sol i 3. Pac c 3 was added to the official list of Hymenoptera venom allergens in 2017 (184).

The venom of the arboreal ant *P. goeldii* contains 15 novel peptides with antibacterial, insecticidal and haemolytic properties that are collectively named Ponericins (185). When subjected to two-dimensional PAGE analysis, approximately 45 protein spots with a molecular weight of 10 to 66 kDa and *pI* between 4 and 10 were observed (62). Immunoblot of the resolved proteins using a serum sample from an allergic patient revealed four IgE-binding protein spots of between 30 and 45 kDa (62).

3.4. *Pogonomyrmex spp*.

The venom of the harvester ant *P. badius* is primarily an aqueous solution with proteins constituting 73% of dry venom (27, 72). Separation of the venom using one-dimensional PAGE revealed the presence of at least nine protein bands (186). In one comparative study, the venoms of *P. maricopa* and *P. rugosus* were found to contain some basic proteins and a pair of highly expressed glycoproteins with a molecular weight of approximately 40 kDa (187). A biochemical study on *P. badius* venom showed that it contains exceptionally high phospholipase A_2, hyaluronidase, acid phosphatase and lipase activity, and also has phospholipase B, alkaline phosphatases and several esterases (18, 79, 188, 189). Histamine and free amino acids were also found in the venom of *P. badius*, while kinin-like activity and kinin agonists were detected in the venom of *P. maricopa* (9, 188, 189).

Physiological studies indicated that the venoms of *P. badius* and *P. barbatus* exhibit strong central and peripheral neurotoxicity in mammals (78, 186). Comparative toxicological analysis in mice showed that the venom of *Pogonomyrmex spp.* appears to be the most toxic Hymenoptera venom (Table 2), and is comparable to that of cobra venom (9, 76). In comparison to other stinging ant species, the venom of *P. badius* is highly haemolytic. Only 4–8 µg/mL venom induced more than 95% haemolysis in a 5% suspension of mouse erythrocytes (78, 79, 181). A further study on the haemolytic components in *P. barbatus* venom described the isolation of a highly basic, 34 amino acid polypeptide, with a *pI* of 10.0

and a molecular weight of 3.5 kDa, which was termed Barbatolysin (190). Another polypeptide with very high toxicity in mice and a *pI* of 8.0 and a molecular weight of about 8 kDa was also reported in this study (190).

Overall, *P. maricopa* and *P. rugosus* venoms exhibit similar immuno-profiles (187), which is consistent with immunological studies of patients sensitized to *P. maricopa* and *P. rugosus* that demonstrate antigenic cross-reactivity to the venom of seven other harvester ant species (73, 191). Thus, an individual sensitized to one species of *Pogonomyrmex* is likely to react to a sting from another species. Cross-reactivity studies of *Pogonomyrmex* whole-body extract (WBE) by immunodiffusion with commercial WBE of IFA, *Camponotus spp*. (carpenter ant), *Formica spp.* (wood ant), and a stinging insect mixture of honeybee and vespids did not observe any cross-reactivity potential (73). The venom allergens in *Pogonomyrmex spp*. have not been characterized thus far (76, 140, 154). The currently known allergens in stinging ant venoms are summarized in Table 3 below.

Table 3. Allergen components of stinging ant venoms

Species	Allergen name (IUIS nomenclature)	Molecular weight (kDa)	Glycosylated	Properties	References
Fire ant (*Solenopsis invicta*)					
	Sol i 1	37	+	Member of phospholipase A1 protein	(155, 156)
	Sol i 2	28	-	Member of pheromone-binding protein family	(159, 160)
	Sol i 3	24	-	Member of antigen 5 protein family	(27, 153)
	Sol i 4	13.3	-	Member of Sol i 2 family	(100, 159)
Jack Jumper ant (*Myrmecia pilosula*)					
	Myr p 1	6.1	-	Monomer peptide	(174, 176)
	Myr p 2	5.6	-	Heterodimer peptide	(174, 176)
	Myr p 3	8.2	-	Homodimer peptide	(174, 177)
Asian needle ant (*Pachycondyla chinensis*)					
	Pac c 3	23	-	Member of antigen 5 protein family	(183, 184)

IUIS: International Union of Immunological Sciences

4. Diagnosis of ant venom allergy

The symptoms of anaphylaxis following an ant sting are comparable to anaphylaxis caused by other stinging Hymenoptera species. The interval between an Hymenoptera sting and symptoms typically occurs within half an hour (100). Symptoms of acute allergic reactions to Hymenoptera sting can manifest in the skin as itching, flushing, urticaria or angioedema; in the gastrointestinal tract as abdominal cramps, nausea, vomiting and diarrhoea; in the respiratory tract as dyspnoea, hoarseness, asthma or pulmonary oedema; and in the cardiovascular system as dizziness, hypotension, arrhythmia, collapse and loss of consciousness (153). These acute symptoms usually disappear within several hours, but delayed or biphasic anaphylactic reactions to Hymenoptera stings have also been reported (192, 193). Lasting morbidity such as myocardial or cerebrovascular infarction following severe reactions as well as fatalities may occasionally occur (4, 6, 194).

Patients with anaphylaxis to the venom of stinging ants or other stinging Hymenoptera should be referred to an allergist. Evaluation of patients presenting with a potential Hymenoptera sting allergy must include a detailed clinical history (19, 195). Effort should be made to identify the offending insect species, but this may present considerable challenges (196, 197). Other useful clinical clues to the diagnosis of Hymenoptera sting allergy may include the description of a typical nest in the vicinity of the sting incident and the development of typical symptoms, e.g. sterile pustules with IFA sting, presence of jumping ants in sandy soiled area with *M. pilosula* sting, piloerection and sweating around the sting site with *Pogonomyrmex spp.* sting, *etc*. Diagnostic testing should only be performed if there is a definitive clinical history of systemic allergic reaction following a Hymenoptera sting (198). The diagnosis of Hymenoptera allergy is determined by correlation of the clinical manifestation of sting reactions with allergen-specific IgE as determined by intradermal and/or serological testing (199).

4.1. Diagnostic tools

4.1.1. Skin testing

Skin testing is one of the 2 primary confirmatory tests for the presence of allergen-specific IgE antibodies that are commonly used for the diagnosis of allergic disease (200), and is the gold standard for the diagnosis of Hymenoptera venom allergy (201). A skin prick or intradermal injection can be used to apply a Hymenoptera venom extract to the skin. However, skin prick testing with Hymenoptera venom is much less sensitive than intradermal injection and is often omitted in favour of intradermal test (195, 202, 203). The test concentrations which provide optimal sensitivity and specificity typically range between 0.001 and 1 μg/mL for the diagnosis of Hymenoptera venom allergy by intradermal testing (202). Venom concentrations higher than 1 μg/mL may give improved sensitivity, but can also cause false-positive results because of local irritative effects (204). If however, higher concentrations of venom extract (e.g. 10 μg/mL) are required to achieve a greater diagnostic accuracy, dialyzed venom extract, which has naturally occurring histamine removed, can be used to reduce these irritative effects (200, 205). Skin testing should be performed at least 6 weeks after the sting reaction to avoid possible false-negative results during the refractory period, in which allergen-specific IgE antibody can be exhausted (201).

Skin testing for venom allergy to IFA uses whole body extract (WBE), which is prepared by homogenising whole insect bodies (206, 207); although venom extract obtained from homogenates of dissected venom apparatuses might be superior (22, 208), it is not commercially available (see section 5 for further discussion on venom extract). Commercial preparations of *S. invicta* and *S. richteri* WBE for skin testing are available from Stallergenes-Greer (Lenoir, NC, USA) and Hollister-Stier Laboratories (Spokane, WA, USA) (24, 68). Purified extract of *M. pilosula* venom, prepared by the Tasmanian Jack Jumper Allergy Program at the Royal Hobart Hospital is available (209, 210), and has been used in intradermal and *in vitro* testing for the diagnosis of *M. pilosula* venom allergy (211-213). WBE of *P. sennaarensis* for skin testing, prepared by Allerbio Laboratory (Varennes en Argonne, France), has been developed for the diagnosis of allergy to *P. sennaarensis* venom (68). Both WBE formulated in glycerine and venom extracts of *P. chinensis* have been used in skin testing and in *P. chinensis* venom allergy research (56, 57, 122, 183). *P. rugosus* WBE

preparations were available from Greer Laboratories, Hollister-Stier Laboratories and Meridian Biomedical Inc. (73).

4.1.2. Serological testing

Serological (*in vitro*) based tests are an alternative diagnostic method commonly employed by physicians when skin testing is not possible, e.g. in patients with severe dermatographism, when patients are unable to discontinue medications with anti-histaminergic activity, *etc*., or when the skin test is not safe to conduct, e.g. during pregnancy (214). Currently available *in vitro* test methods for the diagnosis of Hymenoptera venom allergy are primarily based on allergosorbent assays for the detection of allergen-specific IgE antibodies. The accuracy of these assays may be affected by the activity of the many allergens in each venom, but also by their binding characteristics for the solid phase used in the assay (200, 215).

The radioallergosorbent test (Phadebas RAST, Phadia, Uppsala, Sweden) was the first *in vitro* assay developed for the detection of allergen-specific IgE antibodies (216). Its original method used allergen coupled to a cyanogen bromide-activated paper disc (allergosorbent) to bind allergen-specific antibodies of all isotypes from serum. After a buffer wash to remove unbound serum proteins, bound allergen-specific IgE antibodies were detected with a radiolabelled anti-human IgE (217). With technological advancements, the RAST assay has evolved into autoanalyzer-based assays, such as the ImmunoCAP system by Phadia, *etc*., that mimics the RAST's solid phase chemistry (200). However, unlike RAST these assays do not use radiolabelled anti-human IgE and are calibrated by means of interpolation of response data from a heterologous serum IgE calibration curve that has been referenced to the World Health Organization IgE serum standard 75/502 (200, 218). The most significant advancement of these next-generation assays however, was the development of an encapsulated cellulose-based carrier polymer to which the allergen of interest was covalently coupled (218, 219). This polymer was three-dimensionally configured into the shape of a small cup and called a CAP. Its use in the Phadia ImmunoCAP system improved the allergosorbent's overall IgE-binding capacity, which led to more rapid assay kinetics and enhanced assay sensitivity (217, 220-222). The Phadia ImmunoCAP system is commercially

available for detecting allergen-specific IgE to *S. invicta* WBE (223, 224), and to *M. pilosula* venom (225, 226). In addition to the ImmunoCAP system, an enzyme-linked immunosorbent assay (ELISA) or comparable *in vitro* assay for measurement of allergen-specific IgE has been developed for *S. invicta* (227), *M. pilosula* (225), and *P. chinensis* venom (57). No *in vitro*-based assay is currently available commercially for the diagnosis of *Pogonomyrmex spp*. venom allergy.

4.1.3. Sting challenge test

A deliberate sting challenge using live insects may be considered as a reference method to confirm Hymenoptera venom allergy (228, 229). In this method, the offending insect species is allowed to intentionally inject its venom into the patient's skin under medical supervision (198, 230). However, the sting challenge test is generally not recommended for routine use in the diagnostic workup of a suspected Hymenoptera venom allergy, due to the risk that deliberate stings could boost the already diminished sensitization, cause severe systemic/life threatening allergic reactions, or even re-sensitize the patients to the venom allergens (231). Instead, the sting challenge test is frequently employed in research environments where informed consent, placement of intravenous lines (cannula) pre-challenge and appropriate medical personnel with experience in treating anaphylactic reactions are available (198). In this setting, the sting challenge test is typically used for assessing the effectiveness of Venom Immunotherapy (see following section) and to identify those who are not yet desensitised by the treatment under study. The sting challenge test with live ants has been used to confirm *P. chinensis* venom as the cause of anaphylaxis (124, 232), and to assess the protection offered by Venom Immunotherapy against *M. pilosula* venom allergy (212, 213).

5. Management of anaphylactic reactions to ant venoms

The treatment of ant venom anaphylaxis is similar to the treatment of Hymenoptera venom anaphylaxis or other causes of anaphylaxis, and depends on the manifestations of the reaction. However, the rapid onset of venom-induced anaphylactic reactions requires an

aggressive treatment that must be started early. The acute management of anaphylaxis comprises the prompt use of epinephrine (adrenaline) injection, and potentially, intravenous fluids and/or supplemental oxygen in the presence of hypotension and respiratory symptoms, respectively (233). Cold compresses, antihistamines and analgesics may be used to treat swelling, pruritus, and local pain as required (233). In patients with a high risk of severe anaphylactic reactions from future Hymenoptera stings, a long-term management using a disease-modifying approach called Venom Immunotherapy (VIT) is indicated (193, 229, 234). VIT involves subcutaneous administration of increasing amounts of Hymenoptera venom extract (usually beginning with a dose of 0.001 to 1 µg and gradually increasing during initiation phase to a maintenance dose of 100 µg) for a period of 3 to 5 years to induce and maintain long-term immunological tolerance to the offending venom allergens (229, 235). The following sections will focus on various aspects pertinent to stinging ant VIT.

5.1. Mechanisms of Hymenoptera venom allergy and Venom Immunotherapy

The allergic response to Hymenoptera venom is initiated by an exposure of antigen presenting cell cells (APCs) in the skin, including Langerhans cells in the epidermis and dendritic cells (DCs) in the dermis, to venom allergens after an incident involving Hymenoptera sting(s). Under the influence of the cytokine IL-4, APCs present venom allergens to polarise naïve CD4+ T helper (Th) cells to differentiate to the pro-allergic CD4+ phenotype, T helper type 2 (Th2) cells (236). Interleukin 4 and IL-13 cytokines produced by the activated Th2 cells cause B cells to class switch antibody production to generate allergen-specific IgE (sIgE). This sIgE binds to high-affinity FcεR1 IgE receptors on the surface of inflammatory effector cells (mast cells and basophils) and DCs, as well as to low-affinity FcεR2 IgE receptors on the surface of B cells thus 'priming' these cells (237, 238). Upon subsequent sting exposure(s), venom allergen(s) cross-link receptor-bound sIgE on mast cells and basophils, leading to cell degranulation and the release of inflammatory allergy mediators (histamine, leukotrienes, prostaglandins, *etc*.) and inflammatory cell enzymes (e.g. mast cell tryptase). Additionally, sIgE forms allergen-IgE complexes that more effectively bind to the IgE receptors on the surfaces of DCs and B cells. This further increases

the processing and presentation of venom allergen to naïve Th cells, resulting in enhanced Th2 cell activation and secretion of Th2 cytokines. Eosinophilic activation, maturation, survival and infiltration into tissues occurs in the presence of Th2 cell derived IL-4, IL-5 and IL-13 (236, 237).

VIT works through complex immunological mechanisms, which are not completely understood, but appears to affect the fine balance between allergen-specific regulatory T (Treg) cells and Th2 cells, Th1 cells, or both. VIT is thought to induce immunological tolerance by the generation of allergen-specific Treg cells characterized by production of IL-10 and/or TGF-β. These cytokines modify the response of Th2 cells to directly or indirectly influence effector cells of allergic inflammation (mast cells, basophils and eosinophils) and B cells, suppress sIgE production and induce the production of 'blocking' IgG4 and IgA antibodies against venom allergens (236, 239, 240). These immunological changes are summarized in Figure 3.

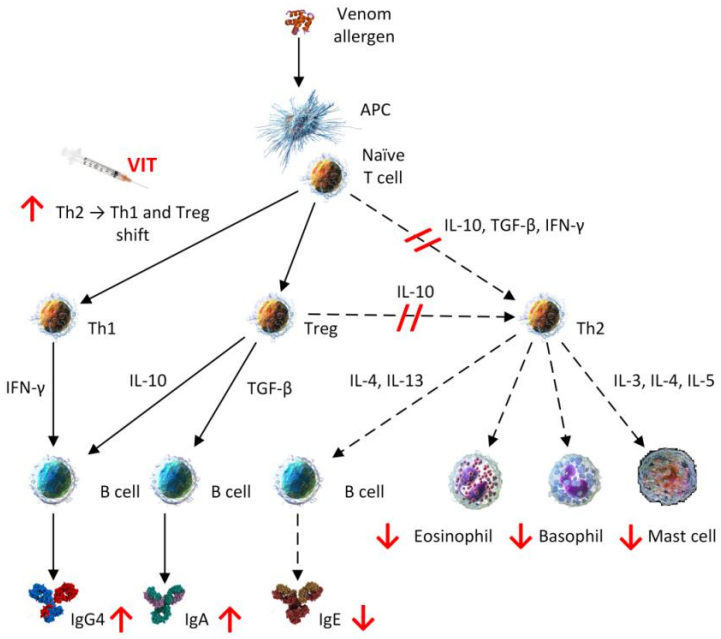

Figure 3. Proposed immunological mechanisms involved in Venom Immunotherapy

Symbols in red indicate immunological changes and CD4+ shift which occur as a result of Venom Immunotherapy. APC: Antigen presenting cell; IFN-γ: Interferon gamma, Ig: Immunoglobulin; IL: Interleukin; TGF-β: Transforming growth factor beta; Th: CD4+ T-helper cell; Treg: Regulatory T cell; VIT: Venom Immunotherapy. Illustrations remain copyright of: www.ebi.ac.uk (allergen); www.123rf.com (antibodies); www.kisspng.com (APC); www.blausen.com (leukocytes).

5.2. Venom collection and purification

Current diagnostic tests for Hymenoptera venom allergy and the preparations used in VIT are based on extracts of the allergen source materials. Consequently, a fundamental prerequisite is the development of appropriate methods to collect venom extracts. Stinging ants on average possess 10 µg of dried venom per individual with the largest ants (e.g. *Myrmecia forficata*) yielding up to 300 µg and the smallest (e.g. IFA) as low as 10 ng of venom proteins (149, 186). In comparison, spiders, scorpions, and snakes typically yield between 1 and 100 mg of venom per species (186). As stinging ants must typically be collected from the their nests in the field, obtaining ant venoms in reasonable quantities and purities may result in practical difficulties (18, 241). A number of methods for Hymenoptera venom collection have been described (207), but the two main methods of collecting venoms from stinging ants are by manual dissection of their venom sac and by electrostimulation (242).

The venom sac dissection (VSD) technique was first introduced in 1964 and has been used for a variety of different arthropods (243). The method of venom collection by VSD begins with the collection of live insects from their nests, before the insects are snap-frozen at –20^{o}C or lower. The frozen insects are then thawed for species identification, quality controlled for parasites, dirt and other contaminants, before the venom sac is removed. The collected venom sacs are lightly crushed, homogenized and filtered to purify the venom (244-246). This basic VSD technique was developed further for commercial purposes by scientists at Vespa Laboratories together with scientists at the Hollister-Stier Laboratories (247). The VSD procedure requires good hand-eye coordination and patience as the venom sacs are extremely fragile and any rupture during dissection will lead to venom loss (248). The obvious disadvantage of VSD is that the ants must be sacrificed. This method has been employed to extract venoms from *Myrmecia* (41, 42, 168, 182, 211), *Pachycondyla* (55, 57, 62), and *Pogonomyrmex* ants (78, 79, 188, 190).

The electrostimulation method was first described by Markovic and Molnar in 1954 for obtaining venom from honeybees (*Apis mellifera*) (202) and was perfected by Benton *et al*. in 1963 (249). The procedure is relatively simple and was adapted to collect venom from other Hymenoptera insects, including vespid and stinging ants (73, 242, 250). The potential

advantages of electrostimulation are that it may give a higher yield of venom if performed *en masse*, is less time consuming and keeps the insect alive for subsequent venom extraction (249). In practice however, electrostimulation is not particularly effective for collecting venom from ants because unlike honeybees, they do not readily release their venom when stimulated (18), however it has been used to collect venoms from IFA (151), *M. pilosula* (176, 210), *M. gulosa* (171), and *Pogonomyrmex* ants (18).

An alternative method of venom collection is to 'milk' the venom from individual live ants into glass rods, capillary tubes, or through membranes (154, 245). The venom is then collected by washing it with an appropriate buffer (248). Small to moderate amounts of relatively pure venom can be collected, but the method is tedious and inefficient for commercial use (244). For safety, it is recommended that ants be chilled and pinned, and handled with the pins while the ants are being 'milked' (154).

After extraction from the source material, the resulting venom extract is clarified to separate solid contaminants from aqueous extract. Typically, a series of graded filters is used for clarification, but other techniques such as dialysis and centrifugation, may also be used. Venom extract is then filtered through a 0.2 µm filter for sterilization and is usually freeze-dried and stored frozen (244).

Problems do exist in the methods of venom collection and venoms may contain small amounts of non-venom proteins (251). The main contaminants of venom obtained using electrostimulation are stinger lancets, mouth secretions, faecal material and body hairs (18, 154). Studies by others suggest that dissected venom extracts contain most of the proteins that are in electrically stimulated venom, but also other proteins that are usually contaminants from the venom sac, including structural and cellular proteins (242, 252). While venom produced by electrical stimulation may give a more genuine representation of the venom components, the method may not be suitable for collecting venom from aggressive Hymenoptera species such as *Myrmecia* ants and vespids (241, 253). Venom collection by this method can be dangerous as the electrically stimulated species will release alarm pheromone and volatile sting substances, causing relevant species in the area to react simultaneously and resulting in uninvolved persons being attacked and stung (154, 253). Although both the electrostimulation and VSD methods have been used in the past to

obtain venom extract from *M. pilosula* (176, 210), venom from this highly aggressive ant species is best collected using the VSD method largely for the reasons described above.

The venom sac of each IFA contains about 40 nanolitre of venom (145). This minute amount of venom can be laboriously collected by either having live ants directly stinging into capillary tubes or by hand-milking venom from dissected venom sacs into micro-capillary pipettes under a dissecting microscope (22, 151). Both methods are extremely laborious and the amount of venom obtained is small. More recently, an alternative method for the extraction of IFA venom has been described, which involves soaking large quantities of fire ants in a dual-phase mixture of non-polar organic solvent and water (254). However, solvent extractions are bound to select for classes of compounds based on their relative solubility and may result in venom losses during the evaporation step (255). A proprietary modified electrical stimulation technique was developed by Vespa Laboratories for the collection of a prototype commercial-grade IFA venom (151, 154); the currently available commercial products are however, still prepared from whole body extracts (202).

5.3. Standardization and stability of ant venom extracts for human use

Comparable to other venomous animals, the synthesis and chemical composition of venoms from various stinging ants vary with time and are influenced by endogenous and exogenous factors (256-261). Likewise, seasonal variation in allergenic composition and activity has also been shown for venom extracts of the IFA, *S. invicta* (262). Accordingly, allergenic composition in different batches of venom extracts is expected to vary and the use of non-standardized venom extracts in the diagnosis of Hymenoptera venom allergy and/or VIT is likely to lead to variations in treatment outcomes. To minimize this potential variability, venom extracts must be prepared and standardized according to regulatory requirements for the manufacture of allergen products (263-266). Although the standardization of allergen products varies for each allergen extract, the process generally consists of the following steps: (i) characterizing prospective reference allergen extract preparations, (ii) identifying major and minor allergenic components in allergen extract, (iii) measuring biological potency of reference allergen extract in human subjects, (iv) developing and validating *in vitro* assays to predict biological potency using reference

allergen extract as the standard, (v) obtaining manufacturing licenses for commercial distribution, and (vi) establishing a Quality System to monitor the regulatory compliance for each manufactured allergen product (215).

The concept of allergen extract standardization, which is one of the cornerstones of allergen immunotherapy, was first introduced by Noon in the early 1900s (267). Allergen extracts are highly heterogeneous mixtures containing multiple allergenic components as active ingredients as well as non-allergenic components (268). Thus, owing to their complex nature, the standardization of allergen extracts is challenging. The modern concept of allergen extract standardization became a reality in the 1970s when the U.S. Food and Drugs Administration (FDA) released the criteria of allergenic potency and allergen composition (269). Central to allergen extract standardization is the use of reference standards (270). Manufacturers of "standardized" allergen products for commercial distribution in the USA must use US reference standards and serum pools specified by the FDA Centre for Biologics Evaluation and Research (CBER) when conducting potency test (263, 266). These standardized products are released and labelled in common bioequivalent allergy units (BAUs) (269), or in micrograms of protein for Hymenoptera venoms (161). In the absence of a US reference standard, the product is exempted by regulation from potency testing and stability study, and is labelled as a "non-standardized" allergen product (263). On the contrary, those manufacturers of allergen products that follow the quality requirements specified in the European Pharmacopoeia monograph on Allergen Products must prepare their own in-house reference (IHR) standard and create their own allergen extract units accordingly (271-273).

Allergen extract batches manufactured for commercial use must be compared with the reference standard using validated qualitative and quantitative *in vitro* assays before batch release (244). Established *in vitro* assay methods for allergen standardization must be able to measure allergenic potency and activity, ensure purity and consistency in allergen composition, and demonstrate the stability of relevant allergens in the extracts (265, 274). Storage conditions, particularly temperature and allergen extract dilution, can affect product quality and potency (275-278). Endogenous proteases, frequently present in allergen extracts, can also lead to the degradation of some allergens during storage (279-

281). Thus, stability studies must be performed to enable proper expiration dating of allergen products (202, 282).

Initial estimations of allergenic extract potency are made by measuring total protein content in the extract using the FDA-approved Lowry (202), or Bradford, ninhydrin, or bicinchoninic acid methods (246, 268). During the commercial development of Hymenoptera venom extracts in the late 1970s, the enzymes phospholipase and hyaluronidase were discovered to be major allergenic proteins in Hymenoptera venoms (202). Functional assays for enzymatic activity confirmed the presence of these enzymes in Hymenoptera venoms and these assays became part of the Hymenoptera venom extract potency testing (202, 266, 283, 284).

The discovery of IgE antibodies by Ishizaka *et al*. in 1966 (285), and the development of immunoassays that measure allergen-specific IgE, led to the use of RAST inhibition and later, ELISA inhibition assay, in potency testing of allergen extracts (266, 286). These assays rely on high-affinity binding interactions between allergens and IgE antibodies derived from human allergic sera (268). The immunoassay approach is, however, highly dependent on the ability of the allergen-specific IgE antibodies to bind the allergens, and variability of the specificity and concentration of the allergen-specific IgE in serum from the selected patients (215, 287). The identification, purification and characterization of relevant allergenic components from various allergen sources have allowed the direct quantification of specific allergen content in allergen extracts (215). Assay methods that are based on monoclonal and polyclonal antibodies that recognize specific allergens, such as the sandwich ELISA assay, have been employed for the standardization of allergen extracts (268, 288). Unlike the RAST and ELISA inhibition assays, this approach is not dependent upon variation in the specificity of patients' allergen-specific IgE antibodies (215).

When specific allergen content cannot be quantified, qualitative assay methods can be employed to monitor the identity, consistency and purity of allergen extracts (215). Additionally, qualitative assays can also serve to complement quantitative analyses (268). In these methods, banding patterns and allergen profiles of allergen extract components, usually produced after electrophoretic separations in porous gels based on molecular weight, charge, or pH differences, are compared to reference standards to establish the

identity and protein composition in the allergen extract batches. Electrophoresis techniques such as isoelectric focusing (IEF), polyacrylamide gel electrophoresis (PAGE), and crossed immunoelectrophoresis (CIE) have been employed to confirm the identity, consistency and purity of allergen extracts (268, 289). Allergen profiles in extracts can be assessed using techniques such as PAGE coupled with immunoblotting, crossed radio-immunoelectrophoresis, or mass spectrometry (287, 290).

Presently, there are two stinging ant-derived products available to diagnose and treat ant venom allergy. The IFA WBE are "non-standardized" commercial products available in the USA, which are neither standardized for potency nor for the presence of the Sol i allergens (161). The concentration of these products is labelled as 1:10 weight/volume of whole-body extracts (244), but an evaluation of three IFA WBE commercial products by crossed immunoelectrophoresis and RAST inhibition assays showed major differences in their antigen contents (291). Furthermore, batch-to-batch variability and stability of these products are not known (202). Purified *M. pilosula* venom (Jack Jumper Ant Venom; JJAV) extract is available in Australia (292). This product, in contrast, is standardized and labelled with concentration of venom protein in mg/mL (210, 293). The standardization procedure, consisting of five complementary assays (Table 4), satisfies the requirements described in the European Pharmacopoeia monograph for Allergen Products (272). While information on batch-to-batch variability of this product is not available, the stability of JJAV extract and diluted preparations containing as low as 0.1 µg/mL of venom protein has been studied (293).

5.4. Efficacy and adverse events associated with stinging ant VIT

The high efficacy of VIT to prevent future sting anaphylaxis caused by social Hymenoptera was shown in multiple systematic reviews and meta-analyses (294-297). To date however, there is only one randomised double-blinded, placebo-controlled trial of VIT against stinging ant allergy, with a deliberate sting challenge test used as a measure of treatment efficacy (153, 292). This trial showed that treatment with *M. pilosula* (Jack Jumper ant; JJA) VIT is highly effective at preventing life-threatening anaphylaxis secondary

to a deliberate sting with live ants. An objective systemic sting reaction rate of less than 5% was observed in patients in the VIT arm compared to 72% in the placebo arm (211).

Table 4. Analytical methods employed in the standardization of JJAV extracts intended for venom allergy diagnosis and treatment

Quality requirements	Method	Acceptance criteria	References
Quantitative assays			
Protein content	Bicinchoninic acid	80–120% of IHR	(210)
Allergenic potency	ELISA inhibition	50–150% of IHR	(210, 272)
Relevant allergen content	HPLC-UV	50–200% of IHR	(176, 210)
Qualitative assays			
Protein profile	SDS-PAGE	Comparable protein banding patterns to IHR	(177, 210)
Allergen profile	SDS-PAGE immunoblot	Comparable IgE-binding patterns to IHR	(174, 210)

As VIT involves injecting patients with a venom extract to which they have specific IgE-mediated allergy, the most common side effects of VIT are systemic allergic reactions, which more often occur during the up-dosing phase of treatment (294, 298). Unlike reactions to wild stings, reactions to venom extract given during VIT are usually mild in nature (e.g. itching, swelling and erythema at injection site, urticaria) due to the gradual up-dosing/slow initiation phase. However, in rare instances reactions to VIT can be so severe (e.g. hypotensive reactions, anaphylaxis) that they require treatment with epinephrine and/or intravenous fluids (298, 299). Recurrent reactions can prolong the time required to achieve maintenance dose. Systemic reactions can also be uncomfortable and/or distressing to patients and when combined with the need for regular visits for treatment continuation, this can reduce patient compliance and premature discontinuation of treatment. For JJA VIT, the risk of adverse reactions is influenced by the rate of initiation and target maintenance

dose (212, 292). Higher rates of systemic reaction occur with fast initiation “ultra-rush” compared to slower initiation “semi-rush” with lower maintenance doses.

5.5. Enhancing the safety and efficacy of VIT using an immunomodulatory adjuvant

Finding new strategies to enhance the safety, efficacy and more compact treatment regimens represent major objectives of current research efforts to improve the delivery of VIT and other forms of Allergen Immunotherapy (AIT) (300). Many innovations and modifications of AIT have been reported (237, 301-303), among these, adjuvants have been shown as encouraging candidates to improve the efficacy and safety of AIT by modulating the non-allergic part of the immune system (Th1 cells) or tolerance-inducing immunological mechanisms (Treg cells) (304). Adjuvants, from the Latin word *adjuvare*, which means ‘to help’ or ‘to enhance’ (305), are all substances that have the potential to enhance the immunogenicity of antigens or allergens. However, adjuvants must themselves be pharmacologically inactive and develop an immunomodulatory effect only in combination with a specific antigen or allergen (304). The use of appropriate adjuvants that skew the immune response toward Th1 and/or Treg and down-regulate the pro-allergic Th2 cell phenotype is therefore a promising strategy to improve the safety and efficacy of AIT. Additionally, a stimulated immune system may require a lower allergen dose and thus reduce AIT-associated side effects by avoiding allergen-IgE complexes (300, 306). However, adjuvants can also cause adverse events, raising clinical concerns about their use (307).

The ideal adjuvants should be cost effective, biodegradable, non-toxic, stable for extended periods of time *in vivo* and induce an appropriate immune response (308). Different adjuvants have been used and/or proposed to enhance the efficacy and safety of AIT and to simplify immunotherapy regimens (309, 310). To date, the most commonly used adjuvant in AIT is Alum, a mixture of aluminium hydroxide, phosphate and sulfate salts, which is used to increase the duration of antigen presentation (302, 311). Alum is however, a relatively weak adjuvant and mainly induces Th2 immunity while stimulating high and persistent levels of IgE antibodies, which limits its use in long-term therapies (300, 312). Additionally, the use of Alum in human vaccines has been linked to persistent intradermal granuloma formation, *macrophagic myofasciitis* and autoimmune/inflammatory syndrome

(311, 313). Recent advances in developing new forms of adjuvants for AIT have focused on the reduction of potential adverse events while modulating the immunogenicity of AIT (310).

Carbohydrates have been recognized to play a critical role in stimulation and regulation of immune responses (311). A major advantage of the use of carbohydrates is their high biocompatibility and low toxicity *in vivo* (314). A wide variety of carbohydrate structures have been tested as adjuvants, with polysaccharides believed to be the most promising carbohydrate structures for vaccine development (314). One potential candidate is the delta inulin-based adjuvant, Advax™. Advax is based on the plant derived fructan inulin (β-D-[2→1] poly(fructo-furanosyl) α-D-glucose; Figure 4) (314, 315). Inulin has no immunological activity when in soluble form, but once formulated into delta inulin microparticles of 1-10 µm size, it shows potent immunomodulatory activity (311, 316-320).

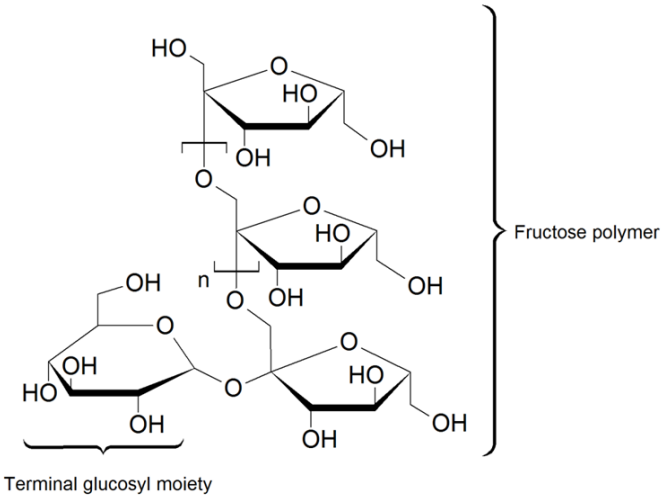

Figure 4. Structure of an inulin polymer

Inulin polymer comprises of a variable length chain of fructose units [n] terminated with a single glucose moiety.

In preclinical studies, Advax enhanced the immunogenicity of a broad range of vaccines including influenza, hepatitis B virus (HBV), Japanese encephalitis, SARS coronavirus, HIV, listeria, RSV, and anthrax (321-323). Furthermore, the safety and efficacy of Advax has been

shown in adult humans when formulated with HBV and influenza vaccines (307, 321, 324). One possible mechanism of action of Advax has been proposed recently. It is thought that Advax may enhance antigen uptake and T- and B-cell responses to co-administered antigen by recruiting and priming APCs (325). A recent study using a combination of Advax with honeybee VIT administered to individuals with honeybee-sting anaphylaxis showed that Advax was safe, enhanced the immunogenicity of honeybee venom, and strongly enhanced allergen-specific IgG4 responses (326). Therefore it is likely that, in combination with stinging ant Venom Immunotherapy (e.g. JJA VIT), Advax may facilitate a desirable immune response to the venom allergen, allowing a reduction in allergen dose, reduced frequency of adverse effects and reduced treatment cost by reducing venom requirements.

5.6. Overview on the development of Jack Jumper ant Venom Immunotherapy

Developments in the field of *M. pilosula* (Jack Jumper) ant Venom Immunotherapy began in 1964 when allergy to *M. pilosula* ant stings was scientifically described for the first time by Dr John Trinca (117). Around the same time, immunotherapy with *M. pilosula* whole body extracts, prepared at the former Commonwealth Serum Laboratories (now CSL Limited), was made available on the Australian Government Pharmaceutical Benefit Scheme and provided to scores of patients from the 1960s onwards (14, 327). It was Dr Struan Sutherland of CSL – the Doyen of envenomation in Australia (328), who first commented in 1980 that pure ant venom preparations would be preferable to whole body extracts for immunotherapy purposes (329). His suggestion stemmed from a personal observation of the failure of whole body extract immunotherapy in protecting anaphylactic reaction during sting challenge and a published case study by Lichtenstein *et al*. of successful immunotherapy with honeybee venom extract (330, 331). This was supported by further research by the same group, in which a double-blind, placebo-controlled trial with vespid venom extract demonstrated the efficacy of Venom Immunotherapy in preventing systemic reactions to insect sting challenge. This study also showed that the preventative activity of whole body extracts was comparable to placebo (332). Despite best efforts, Dr Sutherland reported in 1983 that the proposals to prepare immunotherapy from the venom gland extracts of *M. pilosula* had to be shelved due to the lack of funding (327).

The natural history and the extent of the problem with *M. pilosula* stings was first described by a Tasmanian allergist Dr Paul Clarke in 1986, who conducted a survey on this issue in the Tasmanian population (48). In his report, Dr Clarke also remarked on the unavailability of controlled clinical trial data to assess the prophylactic value of *M. pilosula* whole body extracts, the absence of biochemical and pharmacological analysis on *M. pilosula* venom, and the value of immunotherapy with pure Hymenoptera venom. In his attempts to answer these concerns, Dr Struan Sutherland studied patients' response to immunotherapy with whole body extract preparations produced by CSL over a three-year period. In this research, immunotherapy with whole body extract from crushed ants was found to be ineffective (42, 333), and as a result of this finding the production of *M. pilosula* whole body extracts was ceased in the late 1980s (14, 333). Soon after the problem with *M. pilosula* whole body extract was recognised, Dr Sutherland was awarded an Australian Federal Government grant to collect venom of *Myrmecia* ants for desensitisation purposes (334). Large quantities of venom sac extracts, primarily intended for skin testing of patients, the testing of their sera and their immunotherapy, were accumulated at CSL between 1989 to 1993 (42). The main aim of this initiative was to make *Myrmecia* ants Venom Immunotherapy available for treatment by 1994 (331). In conjunction with these works, detailed studies on *M. pilosula* venom were performed in the 1990s by two groups (328).

Studies on pharmacological and biochemical properties of *M. pilosula* venom were conducted by a group at the Department of Pharmacology at Monash University in Melbourne in collaboration with Dr Sutherland. They found evidence for the presence of histamine, a heat-sensitive haemolytic factor, and eicosanoid-releasing factors (167), and detected phospholipase A_2, phospholipase B, hyaluronidase, acid phosphatase, and alkaline phosphatase activities in *M. pilosula* venom (165). Another group, led by Dr Brian Baldo of the Kolling Institute of Medical Research at The Royal North Shore Hospital in Sydney and in collaboration with Dr Sutherland, aimed to characterise *Myrmecia* venoms and to develop Venom Immunotherapy against *M. pilosula* and *M. pyriformis* (328, 335). Dr John Weiner, an allergist from the Alfred Hospital in Melbourne, was to be in charge of the clinical trials of the Venom Immunotherapy preparations (331). In their initial work, three IgE-binding peptides were identified from the venom of *M. pilosula* (336). Further studies performed by

this research group successfully cloned, sequenced and characterized two IgE-binding peptides, which cDNA they named Myr p 1 and Myr p 2 (337-341). The expressed clones of Myr p 1 and Myr p 2 were found to account for most, but not all, of the venom-specific IgE antibody-binding peptides observed on SDS-PAGE immunoblots of native venom (42). The initiative to produce *Myrmecia* ant Venom Immunotherapy led by Dr Sutherland eventually failed because of technical problems to standardise venom extracts, under-funding and the privatisation of CSL in 1994 (42, 331). Despite the setback, research on the prevalence, natural history and fatalities from systemic allergic reactions to ant venoms in Australia continued to take place in the late 1990s (11, 13, 14, 50). Ultimately, the high incident of systemic allergic reactions and fatalities attributed to *M. pilosula* in Tasmania provided a new stimulus to develop Venom Immunotherapy against this stinging ant (11).

The development of an effective Venom Immunotherapy for *M. pilosula* allergy in Tasmania was instigated by Dr Bryan Walpole and continued by Dr Simon Brown of the Royal Hobart Hospital Department of Emergency Medicine (342). The efficacy of *M. pilosula* Venom Immunotherapy was successfully demonstrated in 2003 in a double-blind, placebo-controlled crossover trial led by Dr Brown (211). In this study, 72% of participants in the placebo arm produced objectively defined systemic reactions to in-hospital sting challenges compared to mild reaction in 3% of participants receiving Venom Immunotherapy. In spite of the success, significant reaction rates of around 34% were also observed during the course of immunotherapy with *M. pilosula* venom extract (211). Following this study, the allergenic components in *M. pilosula* venom were re-examined by the Tasmanian research team (176, 343). Major components in *M. pilosula* venom were characterised by mass spectrometry and it became clear that the original descriptions of the allergenic peptides bases on cDNA sequencing were not entirely correct. A subsequence of the Myr p 2 cDNA was found as a disulfide linked, antiparallel aligned heterodimer consisting of Myr p 2 49 → 74 (des–Gly^{27}-pilosulin 2) and a previously unreported peptide of 2457 Da, while Pilosulin 1, a subsequence of the Myr p 1 cDNA, was found to exist mainly as [Ile^{5}]pilosulin 1 (176). Further analysis of *M. pilosula* venom described novel peptides Pilosulin 4.1 (Myr p 3) and Pilosulin 5 (175, 177), and identified thirteen IgE-binding bands in the venom (174). Pilosulin 3 (Myr p 2) was found to be the only major allergen, whilst [Ile^{5}]pilosulin 1 (Myr p 1) and

pilosulin 4.1 (Myr p 3) were found to be minor allergens (see Chapter 2 for detailed discussion on this topic). There are additional IgE-binding proteins in the venom that require further characterization (174), but the detailed analyses provided an adequate framework for developing a standardization procedure of *M. pilosula* venom extracts for Allergen Immunotherapy that complies with the requirements described in the European Pharmacopoeia (210, 343). The optimal formulation and stability of purified *M. pilosula* venom extracts for use in diagnosis and Venom Immunotherapy was determined (293), and the protocol for *M. pilosula* Venom Immunotherapy was further refined in another randomised controlled trial comparing different treatment regimens (212).

The need for ongoing research to investigate the use of low-dose protocols, immunological adjuvants, and pharmaceutical research to investigate optimal conditions of *M. pilosula* Venom Immunotherapy for distribution and supply to clinicians was recognised by the Australian Society for Clinical Immunology and Allergy (209, 292), which inspired this PhD research. The results of my research and its implications for the delivery of *M. pilosula* Venom Immunotherapy are described in the following chapters.

Chapter 2: In-depth review of Jack Jumper ant venom components

2.1. Introduction

Due to their allergenic potential and impact on human health, studies on JJAV and other *Myrmecia* ant venoms have been performed by many research groups in the last few decades, which resulted in a wealth of available information on this subject. Unfortunately, some of the reported data was found to be conflicting and a source of confusion; for examples see (174, 176, 177). A detailed examination and review of published data was therefore required in order to clarify the correct properties of this highly allergenic venom.

In this foundational work, I performed a comprehensive literature search for published information on *Myrmecia* ant venoms and critically evaluated the available data. I consolidated and cross-referenced our present knowledge on the structural characteristics and mode of action of various bioactive peptides contained within JJAV, and identified the knowledge gaps that require further studies.

2.2. Published manuscript

A manuscript describing this work has been published in *Toxicon*. An electronic reprint is provided.

T. Wanandy, N. Gueven, N.W. Davies, S.G.A. Brown, M.D. Wiese. Pilosulins: A review of the structure and mode of action of venom peptides from an Australian ant *Myrmecia pilosula.* Toxicon. 2015; 98:54-61. (DOI: 10.1016/j.toxicon.2015.02.013)

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Review

Pilosulins: A review of the structure and mode of action of venom peptides from an Australian ant *Myrmecia pilosula*

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ABSTRACT

Myrmecia pilosula is an endemic Australian ant whose sting is a frequent cause of insect allergy in southeast Australia, and several deaths due to *M. pilosula* sting envenomation have been documented. In this review, we discuss the composition and bioactivity of *M. pilosula* venom. In addition to various enzymes and pharmacologically active constituents, the venom contains four families of highly basic low molecular weight peptides trivially named Pilosulins. These peptides are unique and have low structural homology to other Hymenoptera venom peptides. Moreover, *M. pilosula* venom is relatively simple in its composition with 5 predominant peptides making up about 90% by weight. These peptides display cytotoxic, hypotensive, histamine-releasing and antimicrobial activities. Within the *M. pilosula* venom, Pilosulin 3 has been classified as a major allergen and [Ile^5]pilosulin 1 and Pilosulin 4.1 are classified as minor allergens. Several uncharacterised higher molecular weight components with allergenic activities have also been identified. The revised naming of *M. pilosula* venom peptides according to the International Union of Immunological Societies (IUIS) criteria for allergen nomenclature is discussed in this review.

1. Introduction

Myrmecia pilosula species complex (F. Smith, 1858), colloquially known as jack jumper ants, is about 10 mm in length with a black body, long yellow mandibles, yellow legs and very large, anteriorly positioned eyes. They are diurnal foragers, well-known for their exceptional aggressiveness, powerful sting and a characteristic jumping movement (Brown, 1953; Ogata, 1991; Ogata and Taylor, 1991; Sutherland and Tibballs, 2001; Taylor, 1987).

M. pilosula is a member of primitive ants of the genus *Myrmecia*, family Formicidae, order Hymenoptera. There are 89 *Myrmecia* species and sub-species which have been identified so far, 88 of which are endemic to Australia and one rare species, *Myrmecia apicalis* (Emery) is found in New Caledonia (Ogata and Taylor, 1991). *M. pilosula* are commonly found in sandy soiled areas from north of Brisbane, south to Tasmania and west to the vicinity of Denmark in Western Australia (Ogata, 1991; Sutherland and Tibballs, 2001).

M. pilosula is a species complex, consisting of several sibling species with almost identical morphological characteristics (Crosland et al., 1988; Crozier et al., 1995; Taylor, 1991). The species is genetically highly heterogenous and contains at least five karyotypically distinct sub-species which have been scientifically named *Myrmecia croslandi*, *Myrmecia imaii*, *Myrmecia banksi*, *Myrmecia hankinsorum* and *M. pilosula* (Imai and Taylor, 1989; Imai et al., 1994). In some instances, these sibling species have been found to co-exist at a single site (Crosland et al., 1988; Taylor, 1987).

A number of *Myrmecia* ant species are a frequent cause of allergic reactions in humans, with *M. pilosula* the most predominant (Brown et al., 2011; Clarke, 1986; Gilhotra and Brown, 2006;

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Taylor, 1987). In Tasmania where the ants are endemic, 2.7% of the entire population has a history of systemic allergic reactions and approximately half of these are life-threatening anaphylaxis. In rural Victoria, 2.4% of the population has a history of systemic allergic reactions to ant stings, most commonly to *M. pilosula* (Brown et al., 2003a; Clarke, 1986; Douglas et al., 1998). Between 2002 and 2005, 60.8% of the 211 hospitalised cases involving ant sting allergy were due to *M. pilosula* and *Myrmecia pyriformis* (bulldog ant) (Bradley, 2008). In addition, Australian coronial records between 1980 and 1999 attributed six deaths due to anaphylaxis to the venom of *M. pilosula*, *M. pyriformis*, or *Myrmecia forficata* (inchman ant) (Brown et al., 2001; Klotz et al., 2005; McGain and Winkel, 2002).

In comparison to other insect venoms, the venom of *M. pilosula* appears to be particularly allergenic (Taylor, 1987). In individuals with a clinically diagnosed history of honey bee and wasp sting allergy, only 25–50% react to subsequent deliberate sting challenges, whereas the re-sting reaction rate in *M. pilosula* allergic individuals is between 70 and 85% for field stings and 72% for deliberate sting challenges (Brown et al., 2003a, 2003b; van Halteren et al., 1995; Weiner et al., 1995).

In *Myrmecia* species, venom is produced in venom glands, which are formed from modified accessory glands of the female reproductive system. Venom secretions are stored in a venom reservoir (venom sac) and are fed by a duct to the sting bulb (Billen, 1990; Cavill et al., 1964; Robertson, 1968). The amount of venom stored in the venom sac varies between different *Myrmecia* species. Larger species such as *Myrmecia gulosa* may hold as much as 300 μg venom (dry weight), or 0.35% of body weight in its venom sac, while smaller species such as *M. pilosula* hold approximately 40 μg venom (Cavill et al., 1964; Sutherland and Tibballs, 2001). In comparison, *Apis mellifera* (honey bee), *Polistes* spp. (paper wasps) and *Vespula* spp. (yellow jackets) hold approximately 60, 20 and 5 μg of protein in their venom sacs respectively (Hoffman and Jacobson, 1984).

2. *Myrmecia* ant venom composition

The earliest work on *Myrmecia* ant venoms described chemical characteristics of *M. gulosa* venom and showed that the venom contains histamine (2% of dry venom weight) and proteinaceous components that separated electrophoretically into eight bands (Cavill et al., 1964). Venom was shown to contain strong hyaluronidase, heat-labile haemolytic and kinin-like activity (Blum, 1992; Cavill et al., 1964), and was able to inhibit mitochondrial respiration (Ewen and Ilse, 1970).

Analysis of *M. pyriformis* venom also identified a broad range of enzymatic activities including hyaluronidase, phospholipase A_2, phospholipase B, acid phosphatase, and alkaline phosphatase (Lewis et al., 1968; Lewis and de la Lande, 1967; Matuszek et al., 1994; Wanstall and De la Lande, 1974). Furthermore, using isolated rat peritoneal mast cells, potent histamine releasing (Lewis and de la Lande, 1967), and haemolytic and smooth muscle stimulating activity were detected (Wanstall and De la Lande, 1974). Like *M. gulosa*, the venom of *M. pyriformis* also contains histamine (2% of venom dry weight) and a kinin-like substance(s) (Blum, 1992; Cavagnol, 1977; Lewis et al., 1968; Lewis and de la Lande, 1967; Matuszek et al., 1994; Wanstall and De la Lande, 1974).

Analysis of *M. pilosula* venom began in the early 1990s, and it was observed to have very similar pharmacological properties to *M. pyriformis* venom, but there was significantly less enzymatic (i.e. phospholipase B, acid phosphatase, and alkaline phosphatase) activity (Matuszek et al., 1994) and no kinins or acetylcholine content (Matuszek et al., 1992). Analysis using casein-zymography assay did not find proteinase (endopeptidase) activity (Unpublished results, T Wanandy). Histamine accounts for 0.9% of dried venom weight and the venom stimulates inflammatory reactions by releasing cyclooxygenase products (Hodgson, 1997; Matuszek et al., 1992). *M. pilosula* venom also possesses heat-sensitive haemolytic activity, indicating that the component responsible for this property is likely proteinaceous in nature (Matuszek et al., 1992).

The enzymatic and pharmacological components of the three *Myrmecia* venoms are summarised in Table 1.

Contemporary studies on *M. pilosula* venom were started in the early-1990s by Baldo, Donovan et al. and involved electrophoretic separation of the native venom proteins and identification of the IgE-binding components (Ford et al., 1991; Street et al., 1994). The studies led to the cloning, synthesis and characterisation of two

Table 1
Enzymatic and pharmacological components of *Myrmecia* venoms.

	M. gulosa	*M. pyriformis*	*M. pilosula*	References
Enzymatic components				
Acid phosphatase	n.i.	+	+	(Matuszek et al., 1994)
Alkaline phosphatase	n.i	+	+	(Matuszek et al., 1994)
Cholinesterase	0	n.i.	n.i.	(Cavill et al., 1964)
Esterase	n.i.	0	0	(Matuszek et al., 1994)
Hyaluronidase	+	+	+	(Cavill et al., 1964; Lewis and de la Lande, 1967; Matuszek et al., 1994; Wanstall and De la Lande, 1974)
5-nucleotidase	0	n.i.	n.i.	(Cavill et al., 1964)
Phosphodiesterase	n.i.	0	0	(Matuszek et al., 1994)
Phospholipase A_2	n.i.	+	+	(Lewis et al., 1968; Matuszek et al., 1994; Wanstall and De la Lande, 1974)
Phospholipase B	n.i.	+	+	(Matuszek et al., 1994)
Phospholipase C	n.i.	0	0	(Matuszek et al., 1994)
Proteinase	0	n.i.	0	(Cavill et al., 1964)
Pharmacological components				
Acetylcholine	n.i.	n.i.	0	(Matuszek et al., 1992)
Eicosanoid-releasing factors	n.i.	n.i.	+	(Matuszek et al., 1992)
Haemolysins	+	+	+	(Cavill et al., 1964; Lewis and de la Lande, 1967; Matuszek et al., 1992; Wanstall and De la Lande, 1974)
Histamine	+	+	+	(Cavill et al., 1964; Lewis and de la Lande, 1967; Matuszek et al., 1992; Wanstall and De la Lande, 1974)
Histamine-releasing activity	n.i.	+	n.i.	(Lewis and de la Lande, 1967; Wanstall and De la Lande, 1974)
Kinins or kinin-like activity	+	0	0	(Cavill et al., 1964; Lewis and de la Lande, 1967; Matuszek et al., 1992)
Smooth muscle stimulant activity	n.i.	+	n.i.	(Lewis and de la Lande, 1967; Wanstall and De la Lande, 1974)
Mitochondrial respiration inhibitor	+	n.i.	n.i.	(Ewen and Ilse, 1970)

Symbols: + Present; 0 Absent; n.i. Not investigated.

venom allergens and their peptide sub-sequences (Donovan et al., 1996; King et al., 1998; Street et al., 1996; Wu et al., 1998). Further proteomic studies led to a more detailed understanding of allergens found in *M. pilosula* venom and their importance in human anaphylaxis (Davies et al., 2004; Wiese et al., 2007, 2006). Most recently, Japanese and Australian researchers used cDNA from *M. pilosula* species complex and identified several novel allergenic peptides (Inagaki et al., 2004a, 2004b, 2008). The following is a review on the chemistry of *M. pilosula* venom peptide families known by their trivial names as the 'Pilosulins' and our current understanding of their bioactivities.

2.1. Chemistry of Pilosulins

Myrmecia pilosula derived cDNA encoding the Pilosulin 1 peptide family was sequenced by Donovan et al. and named Myr p 1 (Donovan et al., 1993). This peptide is expressed in the venom sac as a precursor peptide containing 112 amino acid residues, and undergoes extensive post-translational modification and was proposed to give rise to a family of six homologous C-terminal peptide sub-sequences containing between 27 and 56 amino acid residues in the final venom (Donovan et al., 1996). The Myr p 1 precursor peptide sequence and proposed sub-sequences are summarised in Table 2. A comparison of Myr p 1 amino acid sequences with other known Hymenoptera venom proteins revealed that it is related to prepromellitin, the precursor of honeybee venom Mellitin (Hoffman, 1996). Myr p 1 shows 42.9% identity and 34% conservative substitutions with residues 32–66 of prepromellitin, which suggests that both proteins may originate from the same gene family.

Myr p 1 derived peptides are highly basic in nature. Donovan et al. predicted that the Myr p 1 precursor 112 residue peptide would have a theoretical p*I* value of 5.95, but the sub-sequences would have an increasingly basic pH as the peptide is further processed. Myr p 1 27 → 112, Myr p 1 37 → 112, and Myr p 1 68 → 112, were predicted to have theoretical p*I* values of 5.23, 9.96, and 10.79, respectively (Donovan et al., 1995). Pilosulin 1 (Myr p 1 57 → 112) is a 56 amino acid residue monomeric peptide with molecular weight 6052 Da and the theoretical p*I* value is 10.45 (Davies et al., 2004; Donovan et al., 1996; Kourie and Shorthouse, 2000). When analysed using circular dichroism, Pilosulin 1 was found to form random coils and have minimal secondary structure. However, in increasingly hydrophobic conditions, approximately one-third of the peptide formed alpha-helix secondary structures (Wu et al., 1998).

In native *M. pilosula* venom, the second most abundant peptide in the venom (after Pilosulin 3) is a variant of Pilosulin 1 (Davies et al., 2004). This peptide has a molecular weight of 6067 Da and it was identified as a variant of Pilosulin 1 where the valine residue at position 5 was replaced by an isoleucine (Table 2); the peptide was therefore named [Ile^5]pilosulin 1 (Davies et al., 2003). Pilosulin 1 itself was found to be a minor component, or in some cases non-existent, in the native venom (Fig. 1). In addition to [Ile^5]pilosulin 1 and Pilosulin 1, other Myr p 1 sub-sequences whose presence in native venom were confirmed by molecular weight and tandem MS data were Myr p 1 68 → 112, Myr p 1 65 → 112 and an oxidized form of [Ile^5]pilosulin 1; these peptide sub-sequence have molecular weights of 4938, 5279, and 6082 Da respectively (Fig. 1) (Davies et al., 2004). Two Myr p 1 sub-sequences, Myr p 1 27 → 112 and Myr p 1 37 → 112, whose existence were predicted by Donovan et al. were not identified in native venom (Davies et al., 2004; Donovan et al., 1996).

A second *M. pilosula* derived cDNA encoding a venom peptide containing 75 amino acid residues was cloned and sequenced (Street et al., 1996). This precursor peptide, named Myr p 2, shares 62% structural homology and a common protein leader sequence with Myr p 1 and differs only by three amino acids in the first 47 residues (Table 2). Similar to Myr p 1, the Myr p 2 precursor peptide undergoes extensive post-translational modification and the fully processed product was proposed to be expressed in the venom as a

Table 2
Proposed precursor peptide sequences and sub-sequences expressed in *M. pilosula* venom.

Peptide Family	Peptide Name	Amino Acid Sequence	Structure	Cysteine Connectivity*
Pilosulin 1	Myr p 1 1 → 56	*MKLSCLLLTLTIIFVLTIVHAPNVEAKDLADPESEAVGFADAFGEADAVGEADPNA*		
	Myr p 1 57 → 112	GLGSVFGRLARILGRVIPKVAKKLGPKVAKVLPKVMKEAIPMAVEMAKSQEEQQPQ	Monomer	
	[Ile^5]pilosulin 1	GLGSIFGRLARILGRVIPKVAKKLGPKVAKVLPKVMKEAIPMAVEMAKSQEEQQPQ	Monomer	
	Myr p 1 65 → 112	LARILGRVIPKVAKKLGPKVAKVLPKVMKEAIPMAVEMAKSQEEQQPQ	Monomer	
	Myr p 1 68 → 112	ILGRVIPKVAKKLGPKVAKVLPKVMKEAIPMAVEMAKSQEEQQPQ	Monomer	
	Myr p 1 71 → 112	RVIPKVAKKLGPKVAKVLPKVMKEAIPMAVEMAKSQEEQQPQ	Monomer	
	Myr p 1 86 → 112	KVLPKVMKEAIPMAVEMAKSQEEQQPQ	Monomer	
Pilosulin 2	Myr p 2 1 → 48	*MKLSCLLLTLAIIFVLTIVHAPNVEAKALADPESDAVGFADAVGEADP*		
	Myr p 2 49 → 75	IDWKKVDWKKVSKKTCKVMLKACKFLG		
Pilosulin 3	Pilosulin 3a	IDWKKVDWKKVSKKTC$_a$KVMLKAC$_b$KFL		
	Pilosulin 3b	LIGLVSKGTC$_c$VLVKTVC$_d$KKVLKQ	Pilosulin 3 is a heterodimer of Pilosulin 3a with Pilosulin 3b	C_a — C_d and C_b — C_c
	Pilosulin 3.1b	LIGLVSKGTC$_c$VLVKTVC$_d$KKVLKQG	Pilosulin 3.1 is a heterodimer of Pilosulin 3a with Pilosulin 3.1b	C_a — C_d and C_b — C_c
	Pilosulin 3.2b	IIGLVSKGTCVLVKTVCKKVLKQG		
Pilosulin 4	Pilosulin 4.1a	FDITKLNIKKLTKATC$_a$KVISKGASMC$_b$KVLFEKKKQE	Pilosulin 4.1 is a homodimer of two Pilosulin 4.1a	C_a — C_a and C_b — C_b
Pilosulin 5	Pilosulin 5a	DVKGMKKAIKEILDC$_a$VIEKGYDKLAAKLKKVIQQLWE	Pilosulin 5 is a homodimer of two Pilosulin 5a	C_a — C_a

*Cysteine Connectivity indicates the position of the cysteines involved in each specific disulfide bonds. For the dimeric peptides, cysteines are highlighted in red and the proposed disulfide bonding patterns are indicated by small subscripted letters. Pilosulin 1 and Pilosulin 2: Precursor peptide sequence is italicised and underlined and mature peptide sequence is shown in capital letters only.

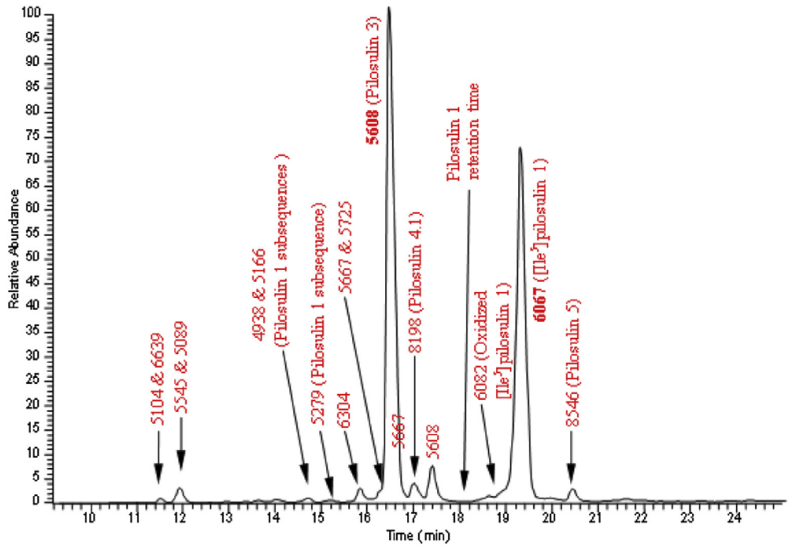

Fig. 1. HPLC-MS total ion chromatogram of *M. pilosula* venom. This sample had Pilosulin 1 exclusively as the isoform [Ile^{5}]pilosulin 1. Molecular weights (in daltons) of some venom peptides shown. Adapted from Davies et al. (2003).

27 amino acid polypeptide (Myr p 2 49 → 75) named Pilosulin 2 with a molecular weight of 3208 Da (Donovan and Baldo, 1997; Donovan et al., 1996). No sequence homology was identified between Myr p 2 49 → 75 and Myr p 1 68 → 112. Donovan et al. predicted Pilosulin 2 would have a theoretical p*I* value of 10.70, and would exist in its native form as a disulfide-linked complex (Donovan et al., 1995, 1996). The Myr p 2 precursor peptide sequence and proposed sub-sequence are summarised in Table 2.

Analysis of *M. pilosula* venom using HPLC-MS by Davies et al. did not find Pilosulin 2 described by Donovan et al., but instead identified a peptide with molecular mass of 5608 Da (Fig. 1) (Davies et al., 2004). This 5608 Da peptide was identified as a heterodimer comprised of two chains with molecular masses of 3155 and 2457 Da. The 3155 Da peptide had an identical amino acid sequence to Pilosulin 2 but without the terminal glycine residue (*i.e.* Myr p 2 49 → 74; des-Gly^{27}-Pilosulin 2) and was named Pilosulin 3a (Table 2). The other chain of the heterodimer peptide had a molecular mass of 2457 Da and was unrelated to Myr p 1 and Myr p 2. This peptide contained 23 amino acid residues and was named Pilosulin 3b. The 5608 Da heterodimer of Pilosulin 3a and Pilosulin 3b, linked in antiparallel fashion by two disulfide bridges, was named Pilosulin 3 (Table 2). In addition to Pilosulin 3 itself, a variant of Pilosulin 3 with a molecular weight of 5667 Da, was also observed by Davies et al. at about 20% of the abundance of Pilosulin 3 (Fig. 1) (Davies et al., 2004; Wiese et al., 2006). This peptide was later found to be a heterodimer comprising Pilosulin 3a and a variant of Pilosulin 3b which had an additional C-terminal glycine, and therefore a molecular weight of 2514 Da. The heterodimer was named Pilosulin 3.1, whilst the variant form of Pilosulin 3b was named Pilosulin 3.1b (Table 2) (Wiese et al., 2006).

At a similar time as Davies et al. conducted the research above, a Japanese research group led by Hidetoshi Inagaki performed characterisation studies on the venom components of *M. banksi*, a member of the *M. pilosula* species complex (Davies et al., 2004; Inagaki et al., 2004a, 2004b). The group obtained cDNA clones encoding novel venom peptides that contained the same common protein leader sequence as Myr p 1 and Myr p 2. The cDNA clones encoded two peptides which Inagaki et al. named 'Pilosulin 3' and 'Pilosulin 4'. The cDNA of 'Pilosulin 3' contains an open reading frame encoding a 74 amino acid precursor peptide and, using the published sequences of the mature forms of Pilosulin 1 and Pilosulin 2, Inagaki et al. predicted that the mature form of their 'Pilosulin 3' exists as a monomer peptide consisting of 24 amino acid residues with a calculated molecular weight of 2520 Da and a theoretical p*I* of 10.41. In addition, compared to Pilosulin 3b they also found that their 'Pilosulin 3' had two cysteine residues and an additional glycine in its C-terminal residue (Inagaki et al., 2004b). Upon further investigation Inagaki's 'Pilosulin 3' was identified as a variant of the peptide that had previously been described by Davies et al. as Pilosulin 3b, but with isoleucine replacing leucine at the N-terminus (*i.e.* [Ile^{1}]pilosulin 3b) and a glycine residue added to the C-terminus (Davies et al., 2004; Inagaki et al., 2004b; Wiese et al., 2006). In order to avoid confusion with the previously named Pilosulin 3 heterodimer, Inagaki's 'Pilosulin 3' monomer was later renamed Pilosulin 3.2b (Inagaki et al., 2008).

Inagaki et al. also described a second novel peptide obtained via cDNA cloning which they named 'Pilosulin 4' (Inagaki et al., 2004b). The cDNA of 'Pilosulin 4' contains an open reading frame encoding an 84 amino acid precursor peptide. The mature form of the peptide, predicted from the sequences of the mature forms of Pilosulin 1 and Pilosulin 2, consists of 36 amino acids with a calculated molecular weight of 4087 Da. The amino acid composition for the mature peptide of 'Pilosulin 4' is similar to Pilosulin 2 and Pilosulin 3a in that it contains two cysteine residues, and both are rich in hydrophobic and basic amino acids. The theoretical p*I* value of the mature peptide of 'Pilosulin 4' is 9.87 (Inagaki et al., 2004b; Wiese et al., 2006). When working with native *M. pilosula* venom Wiese et al. did not observe the presence of 'Pilosulin 4' postulated by Inagaki et al., but rather they found that 'Pilosulin 4' exists as an 8198 Da homodimer peptide consisting of two [Glu^{31}]pilosulin 4 monomers (molecular weight: 4101 Da) linked by two disulfide bridges (Table 2). Wiese et al. named the [Glu^{31}]pilosulin 4 homodimer and monomer as Pilosulin 4.1 and Pilosulin 4.1a, respectively (Wiese et al., 2006).

Using SDS-PAGE and HPLC-MS techniques, Davies et al. and Wiese et al. observed a lesser peptide with molecular mass of

8546 Da as a component in the venom of *M. pilosula* species complex (Fig. 1) (Davies et al., 2004; Wiese et al., 2006). Using a PCR based method, Inagaki et al. independently identified a novel cDNA clone that encoded a new peptide from the mRNA of *M. banksi* which they named Pilosulin 5 (Inagaki et al., 2008). Based on the amino acid sequence data of Pilosulin 5 and tandem mass spectrum analysis of the 8546 Da peptide, Inagaki et al. concluded that the 8546 Da peptide was equivalent to a homodimer of Pilosulin 5 monomeric peptides, which was named Pilosulin 5a, with molecular weight of 4274 Da connected by a single disulfide bridge at cysteine position 15 (Table 2) (Inagaki et al., 2008). Pilosulin 5a is rich in basic and hydrophobic amino acid residues with a calculated p*I* value of 9.16 (Inagaki et al., 2008), and the amino acid sequence between positions 73 to 86 is similar to that of Mastoparan from *Vespula lewisii* venom. Since Mastoparan has an amphiphilic alpha-helix conformation, Inagaki et al. postulated that this region of Pilosulin 5a may adopt the same secondary conformation.

2.2. Mode of action of Pilosulins

The Pilosulins are biologically active peptides that exhibit cytotoxic, hypotensive, histamine-releasing and antimicrobial activities. The cytotoxic activity of Pilosulin 1 was determined by measuring haemolytic activity using human erythrocytes and lymphocytes. Incubation of a 0.5% (v/v) erythrocyte suspension with 40 μM of Pilosulin 1 produced complete lysis after 24 h. Partial lysis was already evident at a concentration of only 1.25 μM (Donovan and Baldo, 1998; King et al., 1998; Wu et al., 1998). Additionally, the authors reported that Pilosulin 1 had similar kinetics but had a more potent cytotoxic effect compared to honeybee venom derived Mellitin. Lysis was rapid (within minutes) and usually complete, but results varied by up to five-fold in leukocytes obtained from different individuals (King et al., 1998). Furthermore, leukocytes were more susceptible to lysis by Pilosulin 1 than granulocytes. Interestingly, the cytotoxic activity of Pilosulin 1 (and Mellitin) was significantly inhibited by the serum protein α-globulin and to a lesser extent by albumin (King et al., 1998). By incubating fragments of synthetic Pilosulin 1 with proliferating Epstein-Barr virus transformed B lymphocytes, it was reported that the NH_2-terminus of Pilosulin 1 was critical for its cytotoxic activity (Wu et al., 1998). The ED_{50} for full length Pilosulin 1 was approximately 0.1 μM, whereas it was 2 μM for Pilosulin 1 (1 → 22) and over 10 μM for Pilosulin 1 (11 → 56), while other peptide segments (23 → 56, 37 → 56, and 47 → 56) did not show any cytotoxic activity. Furthermore, Pilosulin 1 (1 → 22), but not the (23 → 56) peptide segment was shown to interact with unilamellar membranes (Wu et al., 1998). Taken together: (i) Pilosulin 1 is thought to require the NH_2-terminus for its cytotoxic activity, (ii) the NH_2-terminus is required for membrane association rather than for cell lytic activity, and (iii) the domain responsible for cell lysis is likely to extend further than residue 22 (Wu et al., 1998).

Further investigations on the cytotoxicity of Pilosulin 1 were performed by scanning the Swiss-Prot Database (Zelezetsky et al., 2005b). Using a template based on positional residue frequencies in the N-terminal stretch of natural alpha-helical antimicrobial peptides, a segment in Pilosulin 1 corresponding to the 20 N-terminal residues was identified as responsible for the cytotoxic and antimicrobial activities. The investigators also found that the cytotoxic activity of this Pilosulin 1 segment was comparable to Mellitin, confirming earlier results (King et al., 1998; Wu et al., 1998). The peptide segment was chemically synthesised and showed a potent and broad spectrum antimicrobial activity against a number of Gram-positive and Gram-negative bacteria and as well as against the fungus *Candida albicans*. It was postulated that the affinity of Pilosulin 1 peptide for biological membrane and its cytotoxic and antimicrobial activities are due to two common and functionally important properties: (i) a net cationicity of the peptide that facilitates interaction with negatively charged microbial surfaces, and (ii) the ability of the peptides to assume amphipathic, alpha-helical conformation that permit the incorporation into and subsequent lysis of microbial cell membranes (Zelezetsky et al., 2005a).

Analogous to Pilosulin 1, Pilosulin 2 is also associated with cytotoxic activity. Using proliferating Epstein-Barr virus-transformed B lymphocytes, Pilosulin 2 killed more than 50% of the cell population in 5 min at concentrations as little as 15.6 μM (50 μg/mL) (Donovan and Baldo, 1997). However, Pilosulin 2 did not show any haemolytic activity towards red blood cells up to a tested concentration of 80 μM (Wu et al., 1998). Pilosulin 2 demonstrated a hypotensive effect when it was administered to laboratory rats via the tail veins, inducing a 40% decrease in blood pressure and accompanying reduction in heart rate at a dose of 50 μg/kg (Donovan and Baldo, 1997).

In their investigations, Inagaki et al. found that Pilosulin 3.2b exhibited moderate antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* (MIC < 25 μM), slight activity against *Bacillus subtilis* (MIC < 50 μM), but no activity against *Lactococcus garvieae*, *Pseudomonas aeruginosa*, *C. albicans*, and *Saccharomyces cerevisae*. Pilosulin 4.1a exhibited high antimicrobial activity against *E. coli* and *S. aureus* (MIC < 6.25 μM), moderate activity against *P. aeruginosa* (MIC < 25 μM), weak activity against *B. subtilis* (MIC < 50 μM), and no activity against *L. garvieae*, *C. albicans*, and *Saccharomyces cerevisae*. Additionally, Pilosulin 3.2b and Pilosulin 4.1a are devoid of haemolytic activity *in vitro* but exhibit significant and dose-dependent histamine release, as both peptides induced the release of about 50% of the total cellular histamine from rat peritoneal mast cells at a concentration of 5 μM (Inagaki et al., 2004b).

To characterise the biological properties of Pilosulin 5 peptides, a monomeric and dimeric form of Pilosulin 5a that was linked by a single disulfide bridge, as well as two Pilosulin 5a fragments (corresponding to the amino acid sequence 73 → 86 of Pilosulin 5a) which are similar to the amino acid sequence of Mastoparan mature peptide from *V. lewisii* venom were synthesised (Inagaki et al., 2008). Using rat peritoneal mast cells, the Pilosulin 5 dimer and both Pilosulin 5a fragments exhibited a more significant and dose-dependent histamine release than the Pilosulin 5a monomer. Pilosulin 5 did not demonstrate cytotoxic activity, and it was therefore postulated that Pilosulin 5 peptides activate the histamine releasing mechanism through a non-IgE mediated pathway and the activity may reside in the Mastoparan homologous region. Unlike other Pilosulins however, the Pilosulin 5 dimer displayed only weak antimicrobial activity against *E. coli* and *S. cerevisae* and no antimicrobial activity against *S. aureus* and *C. albicans*. The two Pilosulin 5a fragments did not display antimicrobial activity against any of the tested microorganisms.

Current knowledge on the bioactivities of the Pilosulins is summarised in Table 3.

2.3. Higher molecular weight venom components

Although the Pilosulins described above comprise >95% of venom proteins, ten additional bands have been identified in *M. pilosula* venom with approximate molecular weights of 22, 26, 27, 29, 32, 43, 71, 73, 90, and 232 kDa (Wiese et al., 2006, 2008). These unnamed higher molecular weight venom components are presently uncharacterised.

Table 3
Summary of bioactivities of *M. pilosula* venom components.

	Pilosulin 1	Pilosulin 2	Pilosulin 3.2b	Pilosulin 4.1a	Pilosulin 5	Pilosulin 5a
Cytotoxic activity	*	+	0	0	0	
Hypotensive activity		+				
Histamine-releasing activity			+	+	+	+
Antimicrobial activities						
E. coli	*		+	+	+	
P. aeruginosa	*		0	+		
S. aureus	*		+	+	0	
B. subtilis			+	+		
L. garvieae			0	0		
C. albicans	*		0	0	0	
S. cerevisae			0	0	+	

Symbols: + Present; * Activity was present but tested using peptide segment (specific segments are described in the text); 0 Absent.

Table 4
Summary of characterised *Myrmecia pilosula* venom peptides.

Molecular weight	Trivial name	Allergen name	Isoallergen name	Isoform/ variant name	Allergenicity	Notes	Reference(s)
6052 Da	Pilosulin 1	Myr p 1	Myr p 1.01	Myr p 1.0101	Minor allergen	• Identified through cDNA sequencing encoded within Myr p 1 57 → 112 • Found to be a minor constituent in native venom	(Davies et al., 2004; Donovan et al., 1996; Wiese et al., 2007; Wiese et al., 2006)
6067 Da	[Ile^5]pilosulin 1	Myr p 1	Myr p 1.01	Myr p 1.0102	Minor allergen	• Predominant isoform found in native venom • Second most abundant peptide found in native venom	(Davies et al., 2004; Wiese et al., 2007; Wiese et al., 2006)
4938 Da		Myr p 1				• Myr p 1 68 → 112 • Existence identified through molecular weight and tandem MS data	(Davies et al., 2004)
5279 Da		Myr p 1				• Myr p 1 65 → 112 • Existence identified through molecular weight and tandem MS data	(Davies et al., 2004)
6082 Da	Oxidized [Ile^5] pilosulin 1	Myr p 1				• Existence identified through molecular weight and tandem MS data	(Davies et al., 2004)
3208 Da	Pilosulin 2	Myr p 2				• Identified through cDNA sequencing encoded within Myr p 2 49 → 75 • Not observed in native venom, but instead exist in native venom as a component of Pilosulin 3 heterodimer	(Davies et al., 2004; Wiese et al., 2006)
5608 Da	Pilosulin 3	Myr p 2	Myr p 2.01	Myr p 2.0101	Major allergen	• A heterodimer of Pilosulin 3a and Pilosulin 3b linked in anti-parallel fashion through 2 disulfide bridges • The most abundant peptide found in native venom	(Davies et al., 2004; Wiese et al., 2007; Wiese et al., 2006)
3155 Da	Pilosulin 3a	Myr p 2				• des-Gly^{27}-pilosulin 2 is encoded within Myr p 2 49 → 74	(Davies et al., 2004; Wiese et al., 2006)
2457 Da	Pilosulin 3b					• Identified through Edman degradation sequencing as a peptide with 23 amino acid residues	(Davies et al., 2004; Wiese et al., 2006)
5667 Da	Pilosulin 3.1					• Pilosulin 3 heterodimer with an additional C-terminal glycine on the Pilosulin 3b chain • Present at approximately 20% of the Pilosulin 3 abundance	(Davies et al., 2004; Wiese et al., 2006)
2514 Da	Pilosulin 3.1b					• Pilosulin 3b variant with an additional C-terminal glycine	(Wiese et al., 2006)
2520 Da	Pilosulin 3.2b					• Identified through cDNA sequencing • Initially named 'Pilosulin 3'	(Davies et al., 2004; Inagaki et al., 2004a; Inagaki et al., 2004b; Inagaki et al., 2008; Wiese et al., 2006)
8198 Da	Pilosulin 4.1	Myr p 3	Myr p 3.01	Myr p 3.0101	Minor allergen	• Present in native venom as a homodimer of [Glu^{31}]pilosulin 4 linked by 2 disulfide bridges	(Wiese et al., 2007; Wiese et al., 2006)
4087 Da	Pilosulin 4.1a					• Identified through cDNA sequencing • Initially named 'Pilosulin 4' later found to be [Glu^{31}]pilosulin 4 monomer	(Inagaki et al., 2004a; Inagaki et al., 2004b; Wiese et al., 2006)
8546 Da	Pilosulin 5					• Identified through cDNA sequencing • A homodimer of Pilosulin 5a connected by one disulfide bridge on cysteine15	(Davies et al., 2004; Inagaki et al., 2008; Wiese et al., 2006)
4274 Da	Pilosulin 5a					• Pilosulin 5 monomer peptide	(Wiese et al., 2006)

2.4. Allergenic components in M. pilosula venom and revised nomenclature

Using multiple PAGE systems coupled with western blot as the IgE immune-probing method, several allergenic components within the *M. pilosula* venom have been identified (Wiese et al., 2007). More importantly, the study showed that [Ile^5]pilosulin 1, Pilosulin 3 and Pilosulin 4.1 are allergens that are recognised by 33.3%, 77.7% and 16.7% of sera from patients allergic to *M. pilosula* venom, respectively. Additionally, some of the higher molecular weight components (*i.e.* the 22, 26, 32 and 90 kDa bands) are recognised by some individuals who are allergic to *M. pilosula* venom (Wiese et al., 2007), although they cannot be named as allergens as they are presently uncharacterised. Preliminary studies using SDS-PAGE resolved western blot analysis of sera from patients with *M. pilosula* venom allergies with venom from other *Myrmecia* species showed no evidence of cross-reactivity (Unpublished results, M. Wiese).

Allergens are frequently referred to as major and minor depending on whether greater or less than 50% of allergic patients have the corresponding allergen specific IgE (King et al., 1995a). Accordingly, [Ile^5]pilosulin 1 and Pilosulin 4.1 are classified as minor allergens and Pilosulin 3 is a major allergen with the Pilosulin 3a chain as the primary antigenic determinant (Wiese et al., 2007).

To avoid confusion with the identification of *M. pilosula* venom peptides, cDNA clones and their processed forms, a revision to the naming convention of *M. pilosula* venom allergens according to the International Union of Immunological Societies (IUIS) criteria for allergen nomenclature has been proposed (Wiese et al., 2007). Briefly, under this system allergens are designated according to the accepted Linnéan taxonomic name of their source as follows: the first three letters of the genus, space, the first letter of the species, space, and followed by an Arabic numeral which is assigned to individual allergens in the order of their identification. Where isoallergens and variants of an allergen group have been identified, they are designated by suffixes of a period followed by four Arabic numerals. The first two numerals (01–99) refer to a particular isoallergen, and the two subsequent numerals (01–99) refer to a particular variant of a particular isoallergen (King et al., 1995b). Based on these criteria, it was proposed that:

(i) Pilosulin 1 and its predominant isoform/variant [Ile^5]pilosulin 1 should be referred to as Myr p 1.0101 and Myr p 1.0102, respectively;
(ii) Pilosulin 3 heterodimer should be viewed as a revised definition of Myr p 2 and be referred to as Myr p 2.0101; and
(iii) Pilosulin 4.1, which is a sub-sequence of Myr p 3, should be referred as Myr p 3.0101.

Table 4 summarises our knowledge on *M. pilosula* venom components characterised this far.

3. Future studies

To further advance the knowledge on *M. pilosula* venom allergens, research on the higher molecular weight venom components, further identification of IgE-binding epitopes within the venom components, and determination of the two or even three dimensional structures of Pilosulins will be valuable. The expansion of genomics, proteomics and venomics data on insect venoms and venom allergens, and availability of better analytical tools such as the Next-Gen sequencing and Orbitrap MS will assist future studies on this highly allergenic venom.

Ethical statement

This contribution is a review and does not warrant ethical statement.

Role of funding source

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Contributors

The review was conceived, designed and written by T. Wanandy. All authors participated in its revisions and have approved the final form.

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Transparency document

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Chapter 3: Study objective and research questions

3.1. Study objective

The objective of this thesis was to advance the quality, safety and efficacy of pharmaceutical grade venom extracts, prepared from the jumper ant *Myrmecia pilosula*, that are used in *in vitro* and *in vivo* diagnoses of allergen sensitization and in Venom Immunotherapy. I set a number of clinically relevant research questions as a framework for addressing my objective.

3.2. Research questions and hypothesis

Question 1: What are the intrinsic and extrinsic factors influencing batch-to-batch consistency of Jack Jumper ant venom as Pharmaceutical Grade products?

> *Hypothesis: Poor knowledge of intrinsic and extrinsic determinants of batch-to-batch consistency will negatively impact product quality.*

Question 2: Can I establish the identity of high molecular weight components with IgE-binding properties in Jack Jumper ant venom?

> *Hypothesis: Improved knowledge and understanding of venom allergens will aid treatment safety and efficacy, including determination of their cross-reactivity potentials, implementation of component-resolved diagnostics and personalized treatment.*

Question 3: Is there a relationship between IgE recognition to specific venom components and risks for developing side effects or therapeutic failure?

> *Hypothesis: The safety and efficacy of Venom Immunotherapy is strongly correlated with allergen composition in the venom.*

Question 4: Can immune-modulatory adjuvant be incorporated in the formulation for Jack Jumper ant Venom Immunotherapy?

Hypothesis: Adding appropriate immune-modulatory adjuvant to Venom Immunotherapy will improve the safety and efficacy of this treatment modality.

Question 5: Can I establish a robust handling and storage requirements for Jack Jumper ant venom products so to ensure preservation of allergenic activities during clinical use?

Hypothesis: A scientifically solid framework of handling and storage requirements will ensure the quality, safety and efficacy of this treatment modality throughout its product life-cycle.

Part II: RESULTS

Chapter 4: Production and quality of Jack Jumper ant venom as pharmaceutical grade products

4.1. Introduction

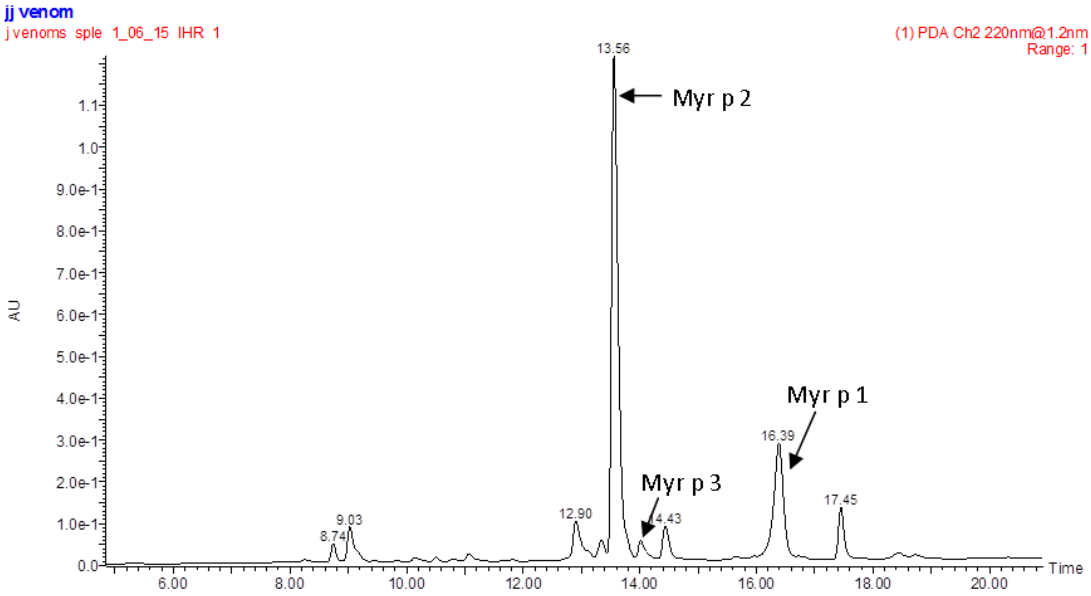
Since 2007, the Tasmanian Jack Jumper Allergy Program at the Royal Hobart Hospital (Australia) has produced purified allergen extracts from JJAV for use in ant venom allergy diagnosis and in VIT. The preparation of JJAV extracts as pharmaceutical grade products (in the form of Active Pharmaceutical Ingredient) is a licensable manufacturing activity, which must be performed under current Good Manufacturing Practice (cGMP) requirements. The products are standardised prior to batch release (210), but batch-to-batch consistency of the extracts is currently not known. Furthermore, detailed requirements for proper handling and storage conditions of the extracts were not fully understood. In this part of the PhD project, I examined the various intrinsic and extrinsic factors that could influence batch-to-batch consistency and quality of JJAV extract as Active Pharmaceutical Ingredients. I also aimed to establish the handling and storage requirements of JJAV extracts to ensure preservation of allergenic activities during usage in *in vitro* and *in vivo* diagnoses of venom sensitization and in VIT.

4.2. Published manuscript

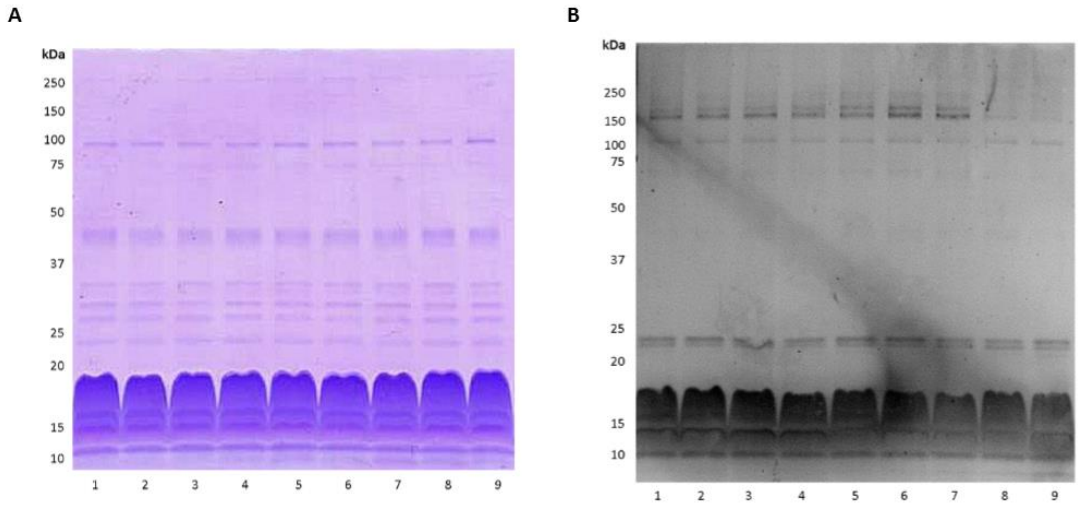
A manuscript describing this work has been published in *Clinical and Experimental Allergy*. An electronic reprint is provided.

T. Wanandy, H.E. Dwyer, L. McLean, N.W. Davies, D. Nichols, N. Gueven, S.G.A. Brown, M.D. Wiese. Factors influencing the quality of Myrmecia pilosula (Jack Jumper) ant venom for use in in vitro and in vivo diagnoses of allergen sensitization and in allergen immunotherapy. Clinical and Experimental Allergy. 2017; 47:1478-1490. (DOI: 10.1111/cea.12987)

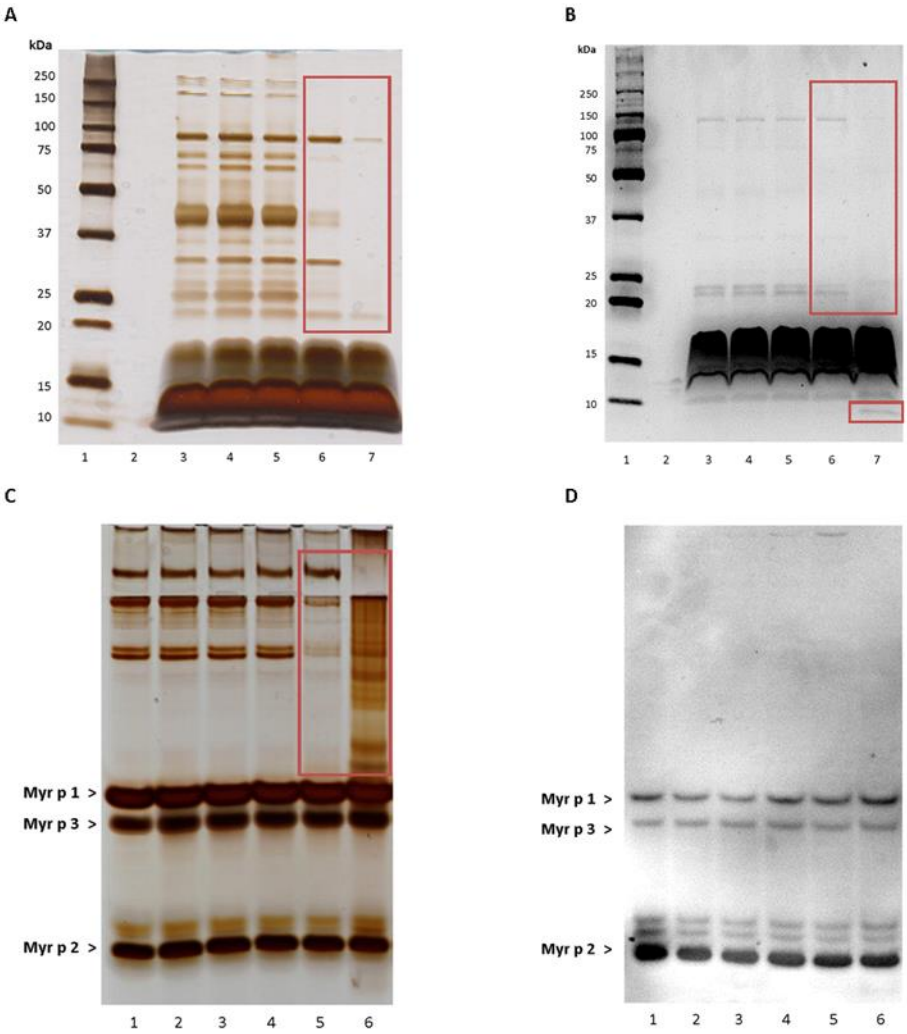
4.3. Supporting information



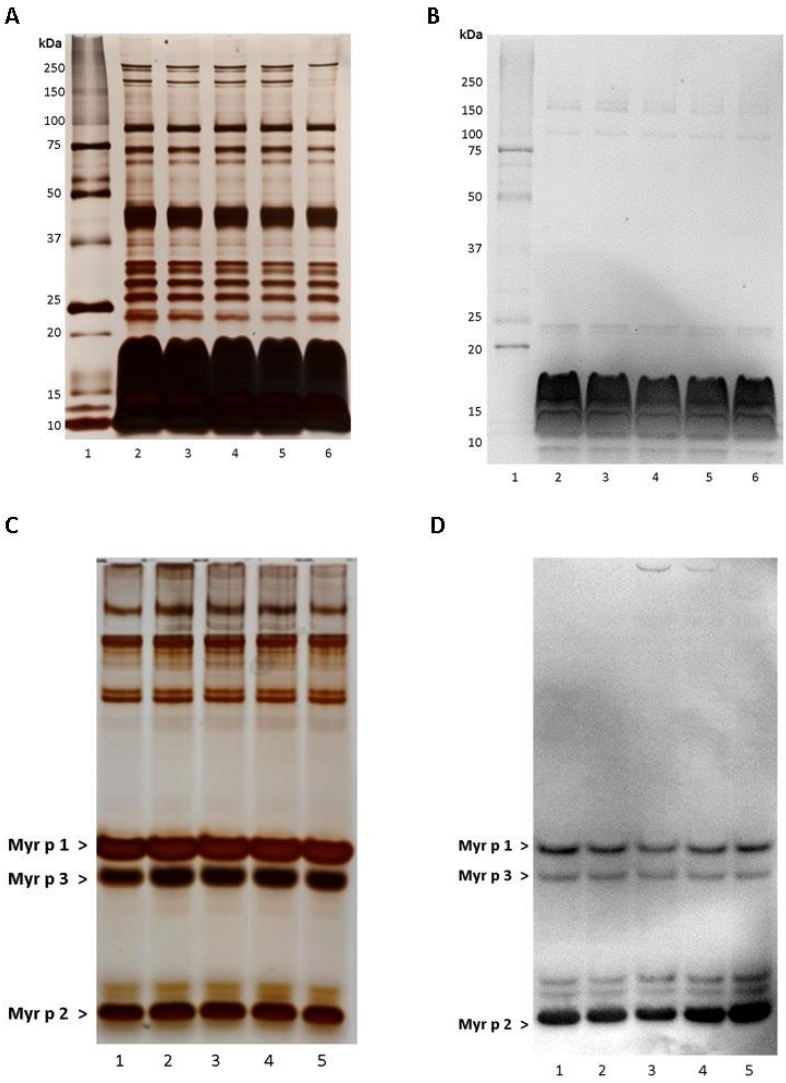
Supplementary Figure 1. A typical HPLC-UV chromatogram of JJAV IHR showing the quantified peaks and retention times corresponding to the allergenic peptides Myr p 1, Myr p 2, and Myr p 3.



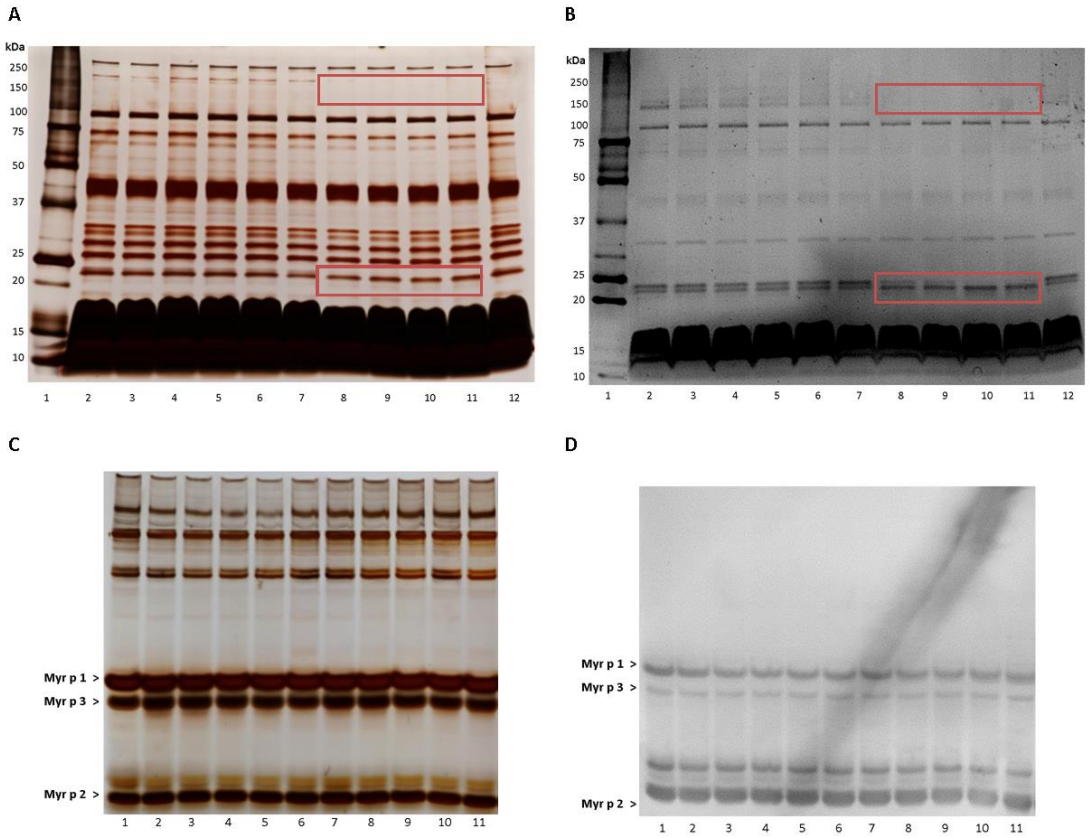
Supplementary Figure 2. Batch-to-batch consistency of JJAV APIs as analysed using SDS-PAGE with Imperial Protein stain (A) and SDS-PAGE Immunoblot (B) methodologies. Lanes: 1. JJAV IHR, 2. API batch 2-02, 3. API batch 2-03, 4. API batch 3-01, 5. API batch 3-02, 6. API batch 3-03, 7. API batch 4-01, 8. API batch 5-01, 9. API batch 5-02.



Supplementary Figure 3. Effects of elevated temperature on the stability of JJAV analysed using SDS-PAGE with silver stain (A), SDS-PAGE Immunoblot (B), AU-PAGE with silver stain (C), and AU-PAGE Immunoblot (D) methodologies. JJAV APIs (1 mg/mL) were incubated at various temperatures for 2 hours. For figures (A) and (B) lanes: 1. MW standards, 2. Empty, 3. Baseline sample, 4. Treatment sample incubated at 20^{o}C, 5. Treatment sample incubated at 40^{o}C, 6. Treatment sample incubated at 60^{o}C, 7. Treatment sample incubated at 80^{o}C. For figures (C) and (D) lanes: 1. JJAV IHR, 2. Baseline sample, 3. Treatment sample incubated at 20^{o}C, 4. Treatment sample incubated at 40^{o}C, 5. Treatment sample incubated at 60^{o}C, and 6. Treatment sample incubated at 80^{o}C.



Supplementary Figure 4. Effects of vial inversion on JJAV analysed using SDS-PAGE with silver stain (A), SDS-PAGE Immunoblot (B), AU-PAGE with silver stain (C), and AU-PAGE Immunoblot (D) methodologies. JJAV APIs (1 mg/mL), protected from light and stored refrigerated at 4^{o}C, were kept in inverted position to ensure full exposure to the inner surface of grey butyl rubber stoppers for various period of time. For figures (A) and (B), lanes: 1. MW standards, 2. Baseline sample, 3. Treatment sample incubated for 6 hours, 4. Treatment sample incubated for 24 hours, 5. Treatment sample incubated for 48 hours, 6. JJAV IHR. For figures (C) and (D), lanes: 1. JJAV IHR, 2. Baseline sample, 3. Treatment sample incubated for 6 hours, 4. Treatment sample incubated for 24 hours, and 5. Treatment sample incubated for 48 hours.



Supplementary Figure 5. Effects of artificial light and storage temperatures on JJAV (1 mg/mL) as analysed by SDS-PAGE with silver stain (A), SDS-PAGE Immunoblot (B), AU-PAGE with silver stain (C), and AU-PAGE Immunoblot methodologies. For figures (A) and (B), lanes: 1. MW standards, 2. Baseline sample, 3. Treatment sample incubated at 4^{o}C with no light for 24 hours, 4. Treatment sample incubated at 4^{o}C with artificial light for 24 hours, 5. Treatment sample incubated at 4^{o}C with no light for 48 hours, 6. Treatment sample incubated at 4^{o}C with artificial light for 48 hours, 7. Baseline sample, 8. Treatment sample incubated at 25^{o}C with no light for 24 hours, 9. Treatment sample incubated at 25^{o}C with artificial light for 24 hours, 10. Treatment sample incubated at 25^{o}C with no light for 48 hours, 11. Treatment sample incubated at 25^{o}C with artificial light for 48 hours, and 12. JJAV IHR. For figures (C) and (D), lanes: 1. JJAV IHR, 2. Baseline sample, 3. Treatment sample incubated at 4^{o}C with no light for 24 hours, 4. Treatment sample incubated at 4^{o}C with artificial light for 24 hours, 5. Treatment sample incubated at 4^{o}C with no light for 48 hours, 6. Treatment sample incubated at 4^{o}C with artificial light for 48 hours, 7. Baseline sample, 8. Treatment sample incubated at 25^{o}C with no light for 24 hours, 9. Treatment sample incubated at 25^{o}C with artificial light for 24 hours, 10. Treatment sample incubated at 25^{o}C

with no light for 48 hours, and 11. Treatment sample incubated at 25^{o}C with artificial light for 48 hours.

Chapter 5: Identification of Jack Jumper ant venom components with IgE-binding capacity and their clinical relevance

5.1. Introduction

Around 80% of patients who are allergic to JJAV are known to recognise Myr p 2, and this major allergen comprises about 50% of JJAV protein (174, 176, 177). However, previous research revealed that many patients are either not susceptible to this allergen, or are also reacting to other allergens such as Myr p 1, Myr p 3, or to the higher molecular weight venom components (>20 kDa) with IgE-binding capacity (174). Importantly, these components are present in JJAV in much lower quantities (343). It is possible that individuals who are allergic to allergens other than the major allergen Myr p 2, and therefore receive much less of it in JJA VIT, are more likely to fail JJA VIT and/or are more or less likely to suffer side effects from JJA VIT. Notably, from the study described in the previous chapter, it became clear that exposure of JJAV to elevated temperature above 40^{o}C degrades the higher molecular weight components of >20 kDa. This could potentially compromise the quality, safety and efficacy of JJAV-derived products, particularly when used in individuals with sensitivity towards these venom components. In this part of the PhD project, I identified IgE-binding components in JJAV, particularly those with a molecular weight of >20 kDa. Additionally, I also aimed to correlate the IgE recognition of JJAV components to clinical data. I looked especially at the efficacy and tolerability/toxicity of this treatment modality, to establish if sub-groups of patients who recognised specific IgE-binding component(s) were more or less likely to experience side effects or therapeutic failure.

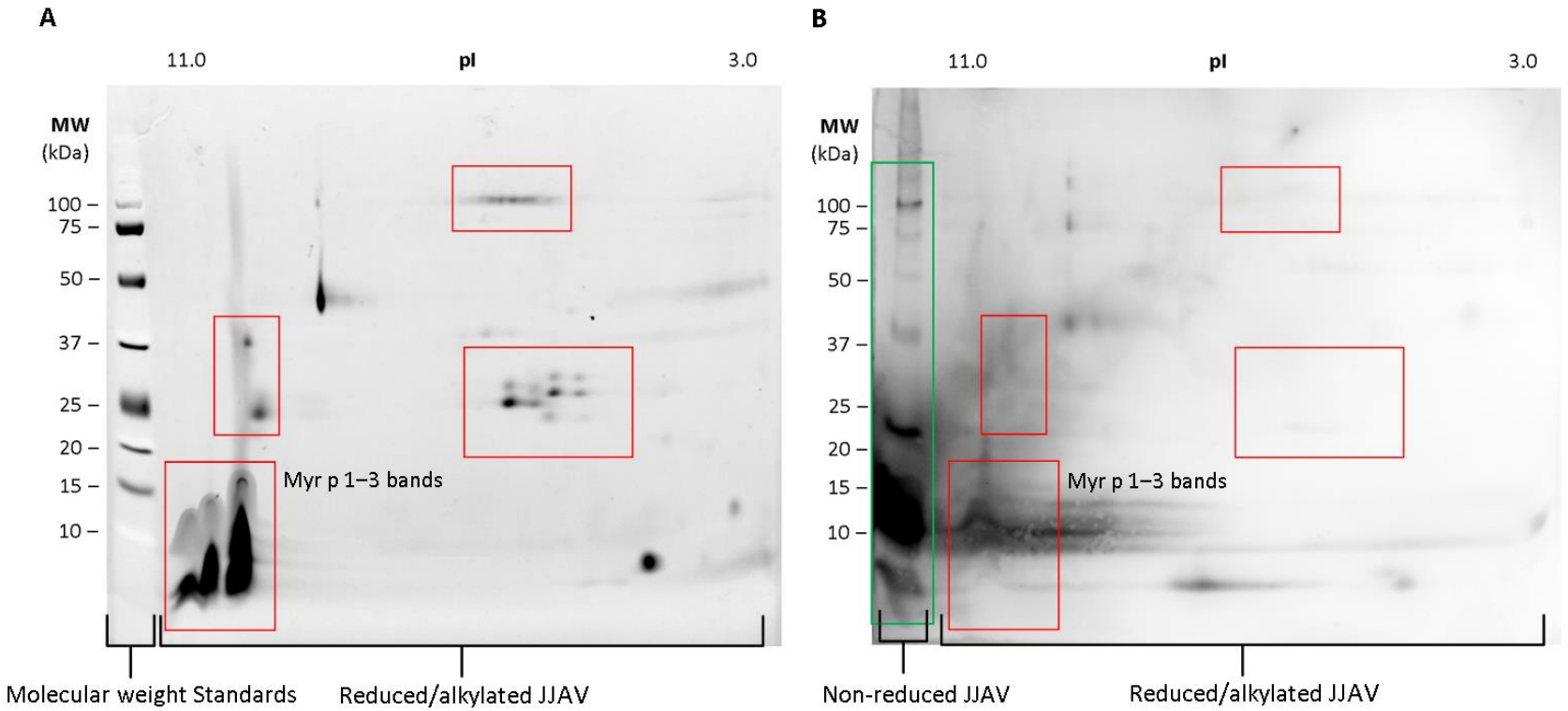
5.2. Published manuscript

A manuscript describing this work has been published in *Clinical and Experimental Allergy*. An electronic reprint is provided.

T. Wanandy, R. Wilson, D. Gell, H.E. Rose, N. Gueven, N.W. Davies, S.G.A. Brown, M.D. Wiese. Towards complete identification of allergens in Jack Jumper (*Myrmecia pilosula*) ant

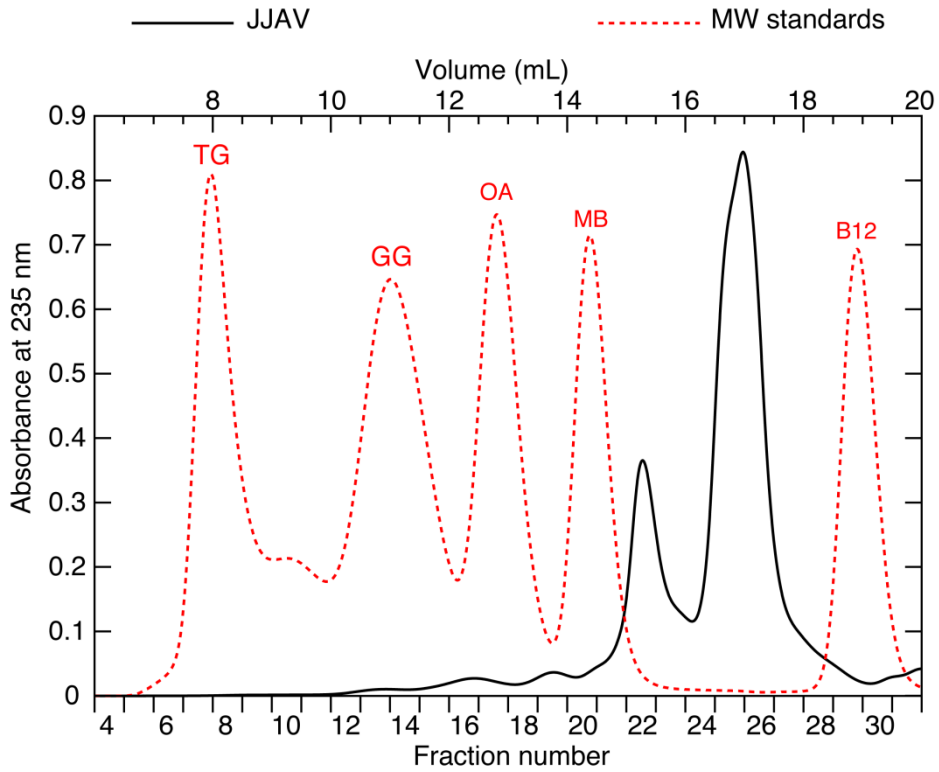
venom and their clinical relevance: An immunoproteomic approach. Clinical and Experimental Allergy. 2018; 48:1222-1234. (DOI: 10.1111/cea.13224)

5.3. Supporting information

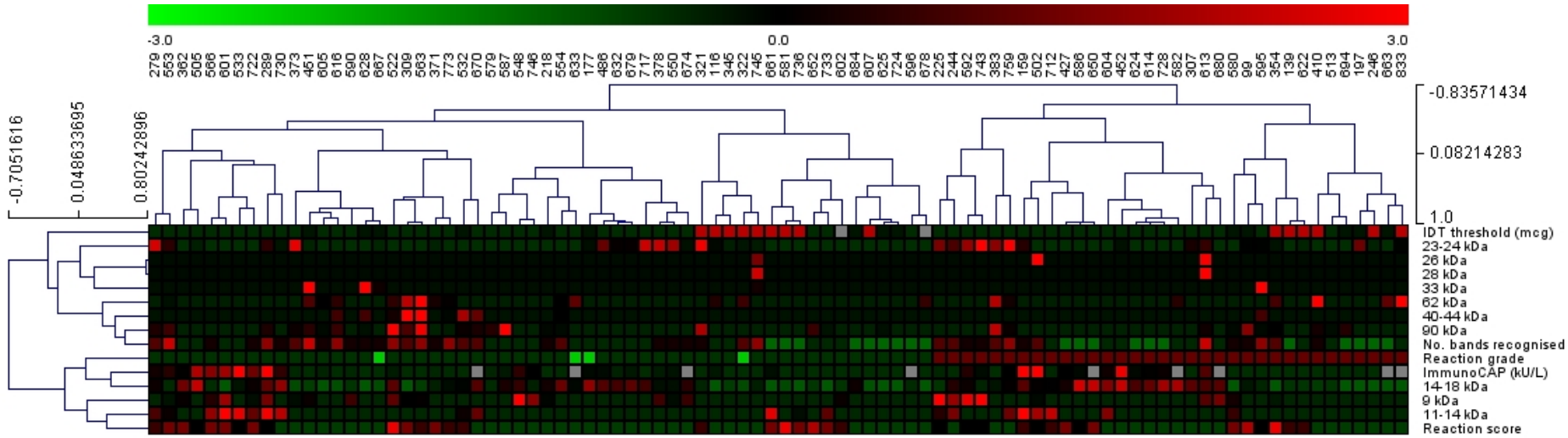


Supplementary Figure 1. Representative profiles of reduced and alkylated JJAV components separated by 2-DE. The first dimension (IEF) was carried out in a non-linear pH 3–11 gradient IPG strip and the second dimension in a 4–12% Bis-Tris SDS-PAGE gel. (A) Second dimension gel was stained with SYPRO Ruby; (B) Second dimension gel was electro-transferred into a PVDF membrane and immunoblotted with a pooled sera from JJAV allergic patients. Areas marked in green and red show appearance of IgE-binding signals in non-reduced JJAV (loaded in the

marker well of the second dimension gel) and disappearance of IgE-binding signals due to reduction/alkylation reactions, respectively. Molecular weight calculation was based on Precision Plus Protein WesternC Standards.



Supplementary Figure 2. Size Exclusion Chromatography profile of JJAV fractionation carried out in Superose® 12 column chromatography. Injections consisted of 0.5 mL of non-reduced JJAV at a concentration of 1 mg/mL. Elution was performed with 50 mM sodium phosphate, 150 mM sodium chloride, pH = 7. Fractions of 0.5 mL were collected at a flow rate of 0.5 mL/min and eluents were detected by absorbance at 235 nm. Molecular weight standards were thyroglobulin (TG, 670 kDa), γ-globulin (GG, 158 kDa), ovalbumin (OA, 44 kDa), myoglobulin (MB, 17 kDa), and vitamin B_{12} (B12, 1.36 kDa).



Supplementary Figure 3. Analysis of individual sera samples to determine clustering patterns in data. Data were normalised across rows and an unsupervised clustering (hierarchical clustering) was performed using a nonparametric Spearman's rank correlation distance metric and average linkage clustering method. A heat-map expression image was created to visualise results. Heat-map colour configuration: grey, black, green, and red squares represent absence of data, unchanged, low (down-regulated), and high (up-regulated) relative expression, respectively. Colour intensity of every single square in the heat-map is directly associated with the intensity of measured value, score or category. Label of dendrogram in vertical direction: 3 digits number represents patient's ID. Label of dendogram in horizontal direction: Reaction score describes patients' reaction profile during VIT categorized numerically as described in Table 1; IDT = intradermal test; Reaction grade = severity of anaphylaxis according to the criteria of Brown (344).

Supplementary Table 1. Peptide evidence for the proteins reported in Table 1

Band no	Protein (Allergen)	Protein ID	Protein Accession Code	Peptide	Unique	-10lgP	Mass	Length	ppm	m/z	z	RT	Area	Scan	#Spec	Start	End	PTM
1	Cu-Zn superoxide dismutase	5563	gi|815812192|ref|XP_012227043.1|	R.HVGDLGNVEASADGVAK.V	Y	75.14	1637.8009	17	0.7	819.9083	2	20.17	9.76E+05	1140	5	79	95	
		5563	gi|815812192|ref|XP_012227043.1|	K.AVCVLQGEPVK.G	N	60.48	1141.6165	11	1.9	571.8166	2	26.76	7.70E+04	1880	1	4	14	
		5563	gi|815812192|ref|XP_012227043.1|	K.VTGEVSGLQK.G	N	48.33	1016.5502	10	0.3	509.2825	2	16.98	2.05E+06	850	3	30	39	
2	Cofilin/actin-depolimerizing factor	2	gi|749756765|ref|XP_011140861.1|	K.YIQATDLSEASEEAVEEK.L	Y	66.08	2010.9269	18	0.9	1006.4716	2	26.3	1.44E+05	1845	1	124	141	
		2	gi|749756765|ref|XP_011140861.1|	R.DAAYDAFLEDLQK.G	Y	60.27	1497.6987	13	2.8	749.8588	2	33.79	2.20E+05	2682	1	46	58	
		2	gi|749756765|ref|XP_011140861.1|	K.MLYSSSFDALKK.S	Y	59.23	1388.7009	12	1.4	695.3587	2	23.68	4.18E+04	1552	1	105	116	
		2	gi|749756765|ref|XP_011140861.1|	K.MLYSSSFDALK.K	Y	51.48	1260.606	11	0.8	631.3107	2	27.18	1.26E+04	1944	1	105	115	
		2	gi|749756765|ref|XP_011140861.1|	K.M(+15.99)LYSSSFDALKK.S	Y	51.03	1404.6959	12	0.2	703.3553	2	22.18	1.11E+04	1385	1	105	116	Oxidation (M)
		2	gi|749756765|ref|XP_011140861.1|	R.YVIFYIKDER.Q	Y	46.43	1344.7078	10	1.6	673.3622	2	26.82	6.32E+04	1904	2	26	35	
		2	gi|749756765|ref|XP_011140861.1|	R.QIDVEVIGPR.D	Y	43.96	1124.6189	10	1.2	563.3174	2	24.9	2.29E+05	1687	1	36	45	
	Phospholipid hydroperoxide glutathione peroxidase isoforms	11166	gi|951523050|ref|XP_014488366.1|	K.ELNELYDEYAESK.G	N	66.63	1601.7096	13	1.2	801.863	2	26.7	2.84E+05	1890	1	54	66	
		11166	gi|951523050|ref|XP_014488366.1|	K.KEQGGLLGNFIK.W	N	55.56	1302.7295	12	1	652.3727	2	25.91	4.02E+04	1801	1	122	133	
		11166	gi|951523050|ref|XP_014488366.1|	K.EQGGLLGNFIK.W	N	53.08	1174.6345	11	1.8	588.3256	2	29.56		2214	1	123	133	
		11166	gi|951523050|ref|XP_014488366.1|	K.IDVNGDKTHPLWSYLK.K	Y	41.43	1884.9734	16	1.1	629.3325	3	26.71	7.18E+04	1891	1	106	121	
		11166	gi|951523050|ref|XP_014488366.1|	K.VKFDLFEK.I	N	35.69	1024.5593	8	1.3	513.2876	2	25.35	1.89E+05	1737	1	98	105	
	Pilosulin 4 (Myr p 3)	11324	gi|51241753|dbj|BAD36780.1|	K.ALADPESDAVGFADAVGEADPFDITK.L	Y	75.92	2620.218	26	4.6	1311.1223	2	37.75	1.16E+05	3159	1	28	53	
3	Muscle-specific protein 20-like	11165	gi|752896490|ref|XP_011266505.1|	R.AGEGLIGLQAGTNK.G	Y	54.48	1327.7096	14	0.4	664.8624	2	23.36	1.73E+04	1587	1	150	163	
		11165	gi|752896490|ref|XP_011266505.1|	K.GATQAGQNFGATR.K	Y	51.21	1277.6112	13	-0.5	639.8126	2	16.48	9.23E+03	850	1	164	176	
		11165	gi|752896490|ref|XP_011266505.1|	K.MMDNLNQFQK.A	Y	48.37	1267.569	10	-0.4	634.7915	2	23.22	6.15E+03	1571	1	73	82	
		11165	gi|752896490|ref|XP_011266505.1|	R.SFTEEQLR.A	Y	34.09	1008.4876	8	0.2	505.2512	2	19.56	8.75E+03	1162	1	142	149	
4	Peptidyl-prolyl cis-trans isomerase-like	11250	gi|749749329|ref|XP_011136874.1|	R.VFFDMTADDKPVGR.I	Y	92.57	1596.7606	14	1	799.3884	2	25.59	3.66E+05	1864	3	51	64	
		11250	gi|749749329|ref|XP_011136874.1|	K.LTHTEPGILSMANAGPNTNGSQFFITSAK.T	Y	69.41	3003.4761	29	2.5	1002.1685	3	29.87		2352	1	135	163	
		11250	gi|749749329|ref|XP_011136874.1|	R.VFFDM(+15.99)TADDKPVGR.I	Y	65.53	1612.7555	14	1.4	807.3862	2	22.46	3.97E+04	1527	3	51	64	Oxidation (M)
		11250	gi|749749329|ref|XP_011136874.1|	K.VVEGMDVVR.K	Y	57.67	1002.5168	9	1	502.2662	2	21.02	8.65E+05	1371	1	177	185	
		11250	gi|749749329|ref|XP_011136874.1|	F.FDMTADDKPVGR.I	Y	49.98	1350.6238	12	2	676.3205	2	25.65	1.66E+04	1870	1	53	64	
		11250	gi|749749329|ref|XP_011136874.1|	N.GSQFFITSAK.T	Y	48.37	1084.5553	10	0.4	543.2852	2	23.56	4.16E+03	1639	1	154	163	
		11250	gi|749749329|ref|XP_011136874.1|	R.KLEAMGSQSGK.T	Y	45.71	1134.5703	11	0.7	568.2928	2	14.07	2.65E+05	765	1	186	196	
		11250	gi|749749329|ref|XP_011136874.1|	K.LTHTEPGILSMAN.A	Y	45.17	1382.6864	13	0.3	692.3507	2	25.24	2.28E+04	1824	1	135	147	
		11250	gi|749749329|ref|XP_011136874.1|	N.AGPNTNGSQFFITSAK.T	Y	43.38	1638.8002	16	-0.1	820.4073	2	24.73	5.86E+03	1767	1	148	163	
		11250	gi|749749329|ref|XP_011136874.1|	K.VVEGM(+15.99)DVVR.K	Y	38.15	1018.5117	9	0.2	510.2632	2	16.98	4.21E+04	974	1	177	185	Oxidation (M)
		11250	gi|749749329|ref|XP_011136874.1|	R.FEDENFK.L	Y	38.12	927.3973	7	-0.1	464.7059	2	18.63	2.32E+04	1120	1	128	134	
	Pilosulin 1 (Myr p 1)	76	gi|730091|sp|Q07932.1|MYR1_MYRPI	K.EAIPMAVEMAK.S	Y	65.59	1188.5883	11	1.4	595.3022	2	25.57	4.18E+06	1861	1	94	104	
		76	gi|730091|sp|Q07932.1|MYR1_MYRPI	K.EAIPMAVEM(+15.99)AK.S	Y	62.95	1204.5831	11	0.5	603.2991	2	22.46	7.46E+05	1526	4	94	104	Oxidation (M)
		76	gi|730091|sp|Q07932.1|MYR1_MYRPI	K.EAIPM(+15.99)AVEM(+15.99)AK.S	Y	54.96	1220.5781	11	-0.4	611.2961	2	19.95	6.49E+05	1256	1	94	104	Oxidation (M)
		76	gi|730091|sp|Q07932.1|MYR1_MYRPI	K.VM(+15.99)KEAIPMAVEMAK.S	Y	40.77	1562.787	14	-0.6	782.4003	2	22.96	4.01E+03	1578	1	91	104	Oxidation (M)
		76	gi|730091|sp|Q07932.1|MYR1_MYRPI	K.EAIPM(+15.99)AVEMAK.S	Y	37.33	1204.5831	11	-0.1	603.2988	2	23.03		1583	1	94	104	Oxidation (M)
		76	gi|730091|sp|Q07932.1|MYR1_MYRPI	K.VMKEAIPMAVEMAK.S	Y	33.06	1546.7921	14	0	774.4033	2	24.71	3.90E+03	1765	1	91	104	
	Pilosulin 3a (Myr p 2)	14082	gi|1911818|gb|AAB50882.1|	K.ALADPESDAVGFADAVGEADPIDWK.K	Y	80.68	2558.1812	25	-0.2	1280.0975	2	36.8		3155	1	28	52	
5	Acidic phospholipase A2, PA4 isoform X2	64	gi|751219056|ref|XP_011162596.1|	R.MVELNADAPFCALYNDR.G	Y	83.97	1940.876	17	4.1	971.4492	2	37.09		3442	1	31	47	
		64	gi|751219056|ref|XP_011162596.1|	A.SVLVADTTM(+15.99)SR.M	Y	61.31	1194.5914	11	0.7	598.3034	2	19.02	6.91E+04	1309	1	20	30	Oxidation (M)
		64	gi|751219056|ref|XP_011162596.1|	A.SVLVADTTMSR.M	Y	59.95	1178.5966	11	1.7	590.3066	2	21.73	8.70E+05	1621	2	20	30	
		64	gi|751219056|ref|XP_011162596.1|	R.IGASVLVADTTMSR.M	Y	55.27	1419.7391	14	3	710.879	2	26.01		2108	1	17	30	
		64	gi|751219056|ref|XP_011162596.1|	V.LVADTTMSR.M	Y	51.47	992.4961	9	0.8	497.2557	2	17.82	1.58E+06	1172	1	22	30	
		64	gi|751219056|ref|XP_011162596.1|	R.MVLGADPRK.V	Y	50.25	985.5378	9	0.5	493.7765	2	17.19	2.35E+04	1109	1	53	61	
		64	gi|751219056|ref|XP_011162596.1|	R.GVIQRMVLGADPR.K	Y	41.53	1410.7765	13	-4.4	706.3924	2	28.96	1.67E+05	2458	1	48	60	
		64	gi|751219056|ref|XP_011162596.1|	R.MVELNADAPF.C	Y	40.59	1105.5114	10	3.1	553.7646	2	32.27	2.79E+04	2849	1	31	40	
		64	gi|751219056|ref|XP_011162596.1|	V.LVADTTM(+15.99)SR.M	Y	38.47	1008.491	9	0	505.2527	2	14.94	2.19E+05	869	1	22	30	Oxidation (M)

Band no	Protein (Allergen)	Protein ID	Protein Accession Code	Peptide	Unique	-10lgP	Mass	Length	ppm	m/z	z	RT	Area	Scan	#Spec	Start	End	PTM
6	Acidic phospholipase A2, PA4 isoform X2	64	gi|751219056|ref|XP_011162596.1|	A.SVLVADTTMSR.M	Y	50.74	1178.5966	11	1.4	590.3064	2	21.8	5.69E+05	1505	2	20	30	
		64	gi|751219056|ref|XP_011162596.1|	A.SVLVADTTM(+15.99)SR.M	Y	48.95	1194.5914	11	0.5	598.3033	2	19.09	7.33E+04	1197	1	20	30	Oxidation (M)
		64	gi|751219056|ref|XP_011162596.1|	R.NGYAEAVSDR.L	Y	48.74	1080.4835	10	0.4	541.2493	2	17.25	5.46E+03	1020	1	199	208	
		64	gi|751219056|ref|XP_011162596.1|	V.LVADTTMSR.M	Y	42.49	992.4961	9	0.7	497.2556	2	17.89	2.76E+05	1076	1	22	30	
		64	gi|751219056|ref|XP_011162596.1|	R.MVELNADAPF.C	Y	37.57	1105.5114	10	3	553.7646	2	32.32	2.38E+04	2701	1	31	40	
		64	gi|751219056|ref|XP_011162596.1|	V.LVADTTM(+15.99)SR.M	Y	36.31	1008.491	9	0.1	505.2528	2	15.1	4.93E+04	844	1	22	30	Oxidation (M)
	Peptidyl-prolyl cis-trans isomerase B	16696	gi|751221702|ref|XP_011164043.1|	R.VIKDFMIQGGDFTK.G	Y	57.66	1597.8174	14	1.6	799.9172	2	26.07	2.09E+04	1988	2	75	88	
		16696	gi|751221702|ref|XP_011164043.1|	K.HYGAGWLSMANAGK.D	Y	51.32	1461.6823	14	-0.9	731.8477	2	24.58	1.14E+04	1818	1	111	124	
		16696	gi|751221702|ref|XP_011164043.1|	K.QTPWLDGR.H	Y	40.12	971.4825	8	0.8	486.7489	2	22.5		1583	1	137	144	
7	Acidic phospholipase A2, PA4 isoform X2	64	gi|751219056|ref|XP_011162596.1|	R.IGASVLVADTTMSR.M	Y	64.55	1419.7391	14	1	710.8776	2	25.95	1.39E+05	2108	1	17	30	
		64	gi|751219056|ref|XP_011162596.1|	A.SVLVADTTM(+15.99)SR.M	Y	56.69	1194.5914	11	1.1	598.3036	2	19.13		1328	1	20	30	Oxidation (M)
		64	gi|751219056|ref|XP_011162596.1|	A.SVLVADTTMSR.M	Y	55.6	1178.5966	11	0.2	590.3057	2	21.83	2.13E+06	1639	1	20	30	
		64	gi|751219056|ref|XP_011162596.1|	R.IGASVLVADTTM(+15.99)SR.M	Y	47.06	1435.734	14	-2.5	718.8725	2	24.13	5.01E+04	1902	1	17	30	Oxidation (M)
8	Phosphoglycerate mutase 1	18120	gi|826414896|ref|XP_012522151.1|	K.DAGYTFDVAHTSVLTR.A	Y	69.57	1751.8478	16	2.5	876.9333	2	27.1	2.94E+04	2198	3	47	62	
		18120	gi|826414896|ref|XP_012522151.1|	K.YGEEQVQIWR.R	Y	61.49	1306.6306	10	-0.3	654.3224	2	25.36	9.21E+04	2001	1	107	116	
		18120	gi|826414896|ref|XP_012522151.1|	R.TLPYWNETIIPQLK.E	Y	60.33	1714.9293	14	2.8	858.4743	2	33.55	7.11E+04	2951	1	164	177	
		18120	gi|826414896|ref|XP_012522151.1|	K.AAMAAVAAQGK.A	Y	59.31	987.5171	11	4.2	494.7679	2	16.51	1.72E+04	992	1	242	252	
		18120	gi|826414896|ref|XP_012522151.1|	R.IIIAAHGNSLR.G	Y	49.91	1163.6775	11	2.2	582.8473	2	19.13	1.36E+04	1295	1	182	192	
		18120	gi|826414896|ref|XP_012522151.1|	R.YADGPKPEEFPK.F	Y	45.07	1376.6611	12	2.5	689.3395	2	19.01	1.78E+04	1281	1	142	153	
		18120	gi|826414896|ref|XP_012522151.1|	R.YADGPKPEEFPKFESLK.L	Y	37.34	1980.9832	17	0.8	661.3355	3	24.39	3.84E+04	1892	1	142	158	
9	Venom allergen 3-like	135	gi|815804483|ref|XP_012222952.1|	S.WDNELETIAQR.W	Y	54.07	1373.6575	11	1.2	687.8369	2	26.66	1.50E+04	2158	1	102	112	
		135	gi|815804483|ref|XP_012222952.1|	W.DNELETIAQR.W	Y	52.42	1187.5782	10	1	594.797	2	23.68	8.53E+04	1820	1	103	112	
10	Annexin B9	6778	gi|752876495|ref|XP_011255648.1|	R.LYASMHGIGTK.D	Y	60.82	1176.5961	11	0	589.3053	2	19.45	2.63E+03	1286	1	261	271	
		6778	gi|752876495|ref|XP_011255648.1|	R.LLVSLVQANR.D	Y	56.09	1111.6713	10	0.4	556.8431	2	25.88		2002	1	158	167	
		6778	gi|752876495|ref|XP_011255648.1|	R.QTFIEYEK.M	Y	39.85	1056.5127	8	0.4	529.2639	2	22.44		1621	1	214	221	
		6778	gi|752876495|ref|XP_011255648.1|	K.AIIDVLTKR.G	Y	32.23	1027.6389	9	0.3	514.8269	2	24.27	3770	1825	1	43	51	
	Apolipoprotein D-like	23	gi|769852441|ref|XP_011637689.1|	K.HEYHYTGQLSVPEPSTPGR.M	Y	61.24	2154.0129	19	0.8	1078.0146	2	22.86	1.18E+04	1668	2	88	106	
		23	gi|769852441|ref|XP_011637689.1|	K.FLGLWYVIQK.T	N	58.82	1265.7172	10	3.4	633.868	2	34.37	1.96E+05	2964	1	40	49	
		23	gi|769852441|ref|XP_011637689.1|	R.TLDQVQVDKIR.Q	Y	48.53	1313.7302	11	-4.4	657.8695	2	20.25	3.12E+04	1376	1	156	166	
11	Hyaluronidase-like	8257	gi|826428058|ref|XP_012527752.1|	R.NGGVPQEGDLKK.H	N	61.61	1240.6411	12	2.4	621.3293	2	15.59	4.08E+06	964	2	99	110	
		8257	gi|826428058|ref|XP_012527752.1|	K.HLEMFQK.H	Y	57.17	931.4586	7	3.5	466.7382	2	18.58	1.56E+06	1318	2	111	117	
12	Elongation factor 1-alpha	9817	gi|795085211|ref|XP_011878235.1|	K.YYVTIIDAPGHR.D	Y	46.02	1403.7197	12	1.8	702.8684	2	23.8	6.58E+03	1727	1	85	96	
		9817	gi|795085211|ref|XP_011878235.1|	R.LPLQDVYK.I	Y	43.68	974.5436	8	0.2	488.2792	2	23.48	3.76E+04	1691	2	248	255	
		9817	gi|795085211|ref|XP_011878235.1|	K.IGGIGTVPVGR.V	Y	42.03	1024.6029	11	0.6	513.309	2	22.63	2.85E+04	1597	1	256	266	
13	Elongation factor 1-alpha isoform	9851	gi|407021090|gb|AFS65405.1|	R.LPLQDVYK.I	Y	43.42	974.5436	8	0.1	488.2791	2	23.53	1.21E+04	1732	1	98	105	
		9851	gi|407021090|gb|AFS65405.1|	K.IGGIGTVPVGR.V	Y	41.15	1024.6029	11	1	513.3093	2	22.67	1.04E+04	1635	1	106	116	
14	Arginine kinase isoform X2	20912	gi|951526129|ref|XP_014467326.1|	K.VSSTLSGLTGELK.G	Y	60.98	1290.7031	13	0.8	646.3594	2	25.67	1.31E+04	1925	1	151	163	
		20912	gi|951526129|ref|XP_014467326.1|	R.LGLTEYQAVK.E	Y	53.1	1120.6128	10	0.6	561.314	2	22.97	2.76E+04	1632	1	330	339	
		20912	gi|951526129|ref|XP_014467326.1|	R.GEHTEAEGGIYDISNK.R	Y	52.67	1718.7747	16	1.4	860.3958	2	19.86	2.21E+03	1291	1	312	327	
		20912	gi|951526129|ref|XP_014467326.1|	K.LIDDHFLFK.E	Y	52.5	1146.6073	9	1.1	574.3116	2	27.28	9.01E+03	2106	1	180	188	
		20912	gi|951526129|ref|XP_014467326.1|	R.LVTAVNEIEK.R	Y	52.34	1114.6234	10	0.2	558.3191	2	21.42	1.84E+04	1462	1	245	254	

Band no	Protein (Allergen)	Protein ID	Protein Accession Code	Peptide	Unique	-10lgP	Mass	Length	ppm	m/z	z	RT	Area	Scan	#Spec	Start	End	PTM
15	Phosphoglycerate kinase	22345	gi|769857915|ref|XP_011640613.1|	K.IGNSLFDEEGAK.I	N	44.63	1278.6091	12	0	640.3118	2	23.64	1.26E+04	1575	1	251	262	
		22345	gi|769857915|ref|XP_011640613.1|	K.SLMDNVVEATTR.G	Y	43.51	1334.65	12	2.1	668.3337	2	28.16	5.54E+04	2070	1	352	363	
		22345	gi|769857915|ref|XP_011640613.1|	R.VDFNVPLK.E	N	37.47	930.5175	8	0.4	466.2662	2	25.83		1807	1	22	29	
		22345	gi|769857915|ref|XP_011640613.1|	R.IVAALDTVK.Y	N	34.25	928.5593	9	1.2	465.2875	2	21.57	7.93E+03	1358	1	39	47	
		22345	gi|769857915|ref|XP_011640613.1|	K.IVNDLLDK.A	N	33.58	928.5229	8	0.3	465.2689	2	22.48	8260	1452	1	263	270	
16	Esterase FE4-like	23704	gi|861647540|gb|KMQ95962.1|	K.DEFGGVVVAVEK.Q	Y	73.33	1247.6398	12	3.2	624.8292	2	28.04	3.32E+05	2149	1	338	349	
		23704	gi|861647540|gb|KMQ95962.1|	R.YAEPPTGQQR.F	N	61.75	1145.5465	10	-0.2	573.7804	2	15.54	2.93E+05	790	1	56	65	
		23704	gi|861647540|gb|KMQ95962.1|	K.APGNLGLKDQVVALR.W	N	61.35	1549.8939	15	0.8	775.9548	2	23.77	4.93E+04	1661	2	172	186	
17	Transferrin	25219	gi|815803678|ref|XP_012222542.1|	R.FFGLPVGVTPAVPTSENPANFR.Y	Y	77.6	2316.1902	22	2.3	1159.105	2	34.54	4.66E+05	2737	2	252	273	
		25219	gi|815803678|ref|XP_012222542.1|	K.LTAMGVLTDINNPEYSAR.E	N	76.93	1963.9673	18	3.6	982.9944	2	30.86	4.12E+05	2329	1	154	171	
		25219	gi|815803678|ref|XP_012222542.1|	K.LTAM(+15.99)GVLTDINNPEYSAR.E	N	72.56	1979.9622	18	2.6	990.9909	2	28	1.03E+05	2010	1	154	171	Oxidation (M)
		25219	gi|815803678|ref|XP_012222542.1|	R.ELTQLGQLGENEK.A	N	69.07	1457.7362	13	-0.3	729.8752	2	23.68	5.21E+05	1529	2	317	329	
		25219	gi|815803678|ref|XP_012222542.1|	K.EADIVAVDPEDMYLAAK.N	N	64.73	1848.8815	17	1.9	925.4498	2	31.72	7.60E+05	2424	1	70	86	
		25219	gi|815803678|ref|XP_012222542.1|	K.TLAVAAPPVSPDEHLK.I	Y	61.26	1643.8882	16	-0.2	822.9512	2	22.91	2.63E+05	1446	3	343	358	
		25219	gi|815803678|ref|XP_012222542.1|	R.ALSTLFDKGCLVGR.W	N	50.6	1478.7915	14	1.8	740.4044	2	29.53	1.49E+04	2182	1	177	190	
		25219	gi|815803678|ref|XP_012222542.1|	R.NVLFKDDVK.E	N	50.1	1076.5865	9	-0.7	539.3002	2	19.81	1.99E+05	1128	1	674	682	
		25219	gi|815803678|ref|XP_012222542.1|	K.RFFGLPVGVTPAVPTSENPANFR.Y	Y	49.15	2472.2913	23	3.9	825.1075	3	31.29	6.56E+04	2377	1	251	273	
		25219	gi|815803678|ref|XP_012222542.1|	R.LFGAWDDKR.N	N	48.83	1106.5509	9	0	554.2827	2	22.53	1.68E+05	1409	1	665	673	
		25219	gi|815803678|ref|XP_012222542.1|	K.YMDVIER.N	N	48.33	924.4375	7	0.4	463.2262	2	21.44	3.46E+05	1301	1	362	368	
		25219	gi|815803678|ref|XP_012222542.1|	K.YM(+15.99)DVIER.N	N	47.25	940.4324	7	3	471.2249	2	18.32	1.20E+04	987	1	362	368	Oxidation (M)
		25219	gi|815803678|ref|XP_012222542.1|	A.PPVSPDEHLK.I	Y	46.95	1117.5768	10	0.6	559.796	2	23	6.18E+03	1456	1	349	358	
		25219	gi|815803678|ref|XP_012222542.1|	K.DIMLLDEK.T	N	45.04	975.4946	8	2.8	488.756	2	28.62	2.03E+05	2082	1	335	342	
		25219	gi|815803678|ref|XP_012222542.1|	M.GVLTDINNPEYSAR.E	N	43.68	1547.7579	14	0.9	774.8869	2	24.57	4.87E+03	1624	1	158	171	
		25219	gi|815803678|ref|XP_012222542.1|	K.DLDINNVQGLR.G	N	42.45	1255.6521	11	0.8	628.8338	2	27		1893	1	122	132	
		25219	gi|815803678|ref|XP_012222542.1|	K.DIM(+15.99)LLDEK.T	N	39.47	991.4896	8	0.7	496.7524	2	26.04	38200	1785	1	335	342	Oxidation (M)
		25219	gi|815803678|ref|XP_012222542.1|	L.AVAAPPVSPDEHLK.I	Y	38.92	1429.7565	14	0.1	715.8856	2	22.99		1455	1	345	358	
		25219	gi|815803678|ref|XP_012222542.1|	R.LFGAWDDK.R	N	34.75	950.4498	8	0.7	476.2325	2	26.03	20900	1784	1	665	672	
		25219	gi|815803678|ref|XP_012222542.1|	L.FGAWDDKR.N	N	33.6	993.4668	8	0	497.7407	2	22.56	8.73E+03	1412	1	666	673	
		25219	gi|815803678|ref|XP_012222542.1|	K.EADIVAVDPEDM(+15.99)YLAAK.N	N	32.41	1864.8763	17	1.9	933.4472	2	28.71	24100	2092	1	70	86	Oxidation (M)
18	Venom dipeptidyl peptidase 4	26382	gi|769837606|ref|XP_011629993.1|	R.VYYLATAPGEPSQR.N	N	73.81	1550.7728	14	2.3	776.3955	2	23.36	3.17E+06	1899	2	420	433	
		26382	gi|769837606|ref|XP_011629993.1|	R.NLYSVPLDASQKPT.C	N	53.5	1531.7882	14	0.8	766.902	2	25.16	3.11E+04	2105	1	434	447	
		26382	gi|769837606|ref|XP_011629993.1|	K.MLFEIYR.N	N	47.12	970.4946	7	2.5	486.2558	2	28.77	1.13E+06	2533	1	596	602	
		26382	gi|769837606|ref|XP_011629993.1|	K.M(+15.99)LFEIYR.N	N	44.45	986.4895	7	3.3	494.2537	2	26.46	1.04E+05	2255	2	596	602	Oxidation (M)
		26382	gi|769837606|ref|XP_011629993.1|	F.EYDHYITTNR.R	Y	38.58	1310.5891	10	3.5	656.3041	2	23.12	1.56E+04	1871	1	568	577	
19	Pilosulin 1 (Myr p 1)	77	gi|1911819|gb|AAB50883.1|	K.EAIPMAVEMAKSQEEQQPQ	Y	76.02	2142.9924	19	1.6	1072.5052	2	26.51	4.47E+04	2105	1	94	112	
		77	gi|1911819|gb|AAB50883.1|	K.EAIPMAVEM(+15.99)AK.S	Y	72.35	1204.5831	11	2.3	603.3002	2	22.51	5.30E+06	1612	2	94	104	Oxidation (M)
		77	gi|1911819|gb|AAB50883.1|	K.EAIPM(+15.99)AVEM(+15.99)AK.S	Y	68.04	1220.5781	11	0.2	611.2964	2	20.03	8.67E+05	1325	2	94	104	Oxidation (M)
		77	gi|1911819|gb|AAB50883.1|	K.EAIPMAVEMAK.S	Y	60.18	1188.5883	11	1.7	595.3024	2	25.52	6.74E+07	1981	2	94	104	
		77	gi|1911819|gb|AAB50883.1|	K.EAIPM(+15.99)AVEMAK.S	Y	57.66	1204.5831	11	2.1	603.3001	2	23.73	3.89E+06	1757	1	94	104	Oxidation (M)
		77	gi|1911819|gb|AAB50883.1|	K.VM(+15.99)KEAIPMAVEMAK.S	Y	56.19	1562.787	14	2.2	782.4025	2	22.99	6.81E+04	1669	2	91	104	Oxidation (M)
		77	gi|1911819|gb|AAB50883.1|	A.IPMAVEMAK.S	Y	55.17	988.5085	9	3.1	495.2631	2	25.62	1.29E+05	1995	1	96	104	
	Pilosulin 5	34801	gi|161788856|dbj|BAF95069.1|	K.AIKEILDCVIEK.G	Y	70.03	1372.7635	12	3.2	687.3912	2	30.31	1.95E+05	2594	1	62	73	
		34801	gi|161788856|dbj|BAF95069.1|	K.EILDCVIEK.G	Y	48.95	1060.5474	9	2.4	531.2822	2	31.12	3.26E+05	2696	1	65	73	
		34801	gi|161788856|dbj|BAF95069.1|	K.KVIQQLWE	Y	43.97	1042.5811	8	3.1	522.2994	2	24.88	3.21E+06	1900	1	84	91	

Supplementary Table 2. Correlation analysis of recognition of JJAV IgE-binding bands

		Calculated molecular weight of IgE-binding band (kDa)									
		9	11–14	14-18	23–24	26	28	33	40–44	62	90
IDT threshold	**r =**	-0.262	-0.271	-0.493	NS	NS	NS	0.210	NS	NS	NS
	***P* =**	0.0137	0.0107	<0.0001				0.0491			
ImmunoCAP	**r =**	0.270	0.486	0.564	NS	NS	NS	-0.254	-0.224	-0.269	-0.263
	***P* =**	0.0147	<0.0001	<0.0001				0.0221	0.0445	0.0154	0.0176

IDT = intradermal test; r = Spearman's correlation coefficient; NS = not significant; Significance < 0.05

Chapter 6: Development and preclinical evaluation of Jack Jumper ant Venom Immunotherapy with *Advax™* adjuvant

6.1. Introduction

The relative difficulty in obtaining pharmaceutical grade ant venom extracts and the increase in demand to treat patients with JJAV allergy within Tasmania and interstate dictates the need to improvise our approach to treat future patients who are severely affected by this highly allergenic ant venom. In this part of the PhD project, I aimed to improve the safety and efficacy of JJA VIT by using lower doses of venom extracts combined with *Advax™* adjuvant. Published clinical studies using *Advax™* in combination with various vaccines (e.g. Hepatitis B, influenza, Japanese encephalitis virus, *etc*.) and more recently with Honeybee VIT (HB VIT) were very promising (321, 323, 326). *Advax™* appears to exhibit a dual effect: (i) it modifies immune responses to provide a more protective effect by enhancing the production of antigen-specific IgG and in the HB VIT study it suppresses the production of allergen-specific IgE, and (ii) it exhibits a "dose-sparing" response allowing lower doses of antigen to be used (326, 345-347). Thus, I hypothesized that co-administration of JJA VIT with *Advax™* would improve the recognition and generation of a protective immune system response towards JJAV allergens and reduce the JJA VIT dose required to achieve the maintenance phase. Improving the immunogenicity of JJAV in the presence of *Advax™* may potentially translate into reduced treatment side effects, assist with the "frequent reactors" to successfully achieve the treatment maintenance phase, and could overall reduce in JJAV requirements and production costs. In this part of my PhD project, I assessed the optimal formulation and physico-chemical and microbiological stability of JJA VIT with and without *Advax™* stored in plastic syringes. Additionally, I evaluated the immunogenicity of low-dose JJA VIT formulated with and without *Advax™* in mice.

6.2. Published manuscript

A manuscript describing this work has been published in the *Journal Pharmaceutical and Biomedical Analysis*. An electronic reprint is provided.

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Pharmaceutical and preclinical evaluation of Advax adjuvant as a dose-sparing strategy for ant venom immunotherapy

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ABSTRACT

A major challenge in broader clinical application of Jack Jumper ant venom immunotherapy (JJA VIT) is the scarcity of ant venom which needs to be manually harvested from wild ants. Adjuvants are commonly used for antigen sparing in other vaccines, and thereby could potentially have major benefits to extend JJA supplies if they were to similarly enhance JJA VIT immunogenicity. The purpose of this study was to evaluate the physicochemical and microbiological stability and murine immunogenicity of low-dose JJA VIT formulated with a novel polysaccharide adjuvant referred to as delta inulin or Advax™. Jack Jumper ant venom (JJAV) protein stability was assessed by UPLC-UV, SDS-PAGE, SDS-PAGE immunoblot, and ELISA inhibition. Diffraction light scattering was used to assess particle size distribution of Advax; pH and benzyl alcohol quantification by UPLC-UV were used to assess the physicochemical stability of JJAV diluent, and endotoxin content and preservative efficacy test was used to investigate the microbiological properties of the adjuvanted VIT formulation. To assess the effect of adjuvant on JJA venom immunogenicity, mice were immunised four times with JJAV alone or formulated with Advax adjuvant. JJA VIT formulated with Advax was found to be physicochemically and microbiologically stable for at least 2 days when stored at 4 and 25 °C with a trend for an increase in allergenic potency observed beyond 2 days of storage. Low-dose JJAV formulated with Advax adjuvant induced significantly higher JJAV-specific IgG than a 5-fold higher dose of JJAV alone, consistent with a powerful allergen-sparing effect. The pharmaceutical data provides important guidance on the formulation, storage and use of JJA VIT formulated with Advax adjuvant, with the murine immunogenicity studies providing a strong rationale for a planned clinical trial to test the ability of Advax adjuvant to achieve 4-fold JJAV dose sparing in JJA-allergic human patients.

1. Introduction

Rapid onset, systemic allergic reactions to Jack Jumper ant (*Myrmecia pilosula*, JJA) sting represent a significant public health problem in the south-eastern and south-western part of Australia, affecting up to 3% of the population [1]. Immunotherapy for the prevention of stinging insect anaphylaxis involves the administration

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0731-7085/

of increasing doses of purified insect venom to induce clinical tolerance, as reflected by a progressive reduction in venom-specific IgE and increase in IgG4 [2]. JJA venom immunotherapy (VIT) involving a large maintenance dose of 100 μg of venom has been shown to be highly effective at preventing JJA sting anaphylaxis, reducing the risk of severe systemic sting reactions by 95% [3]. Real-world experience shows efficacy comparable with yellow jacket and better than honeybee VIT [4]. The applicability of JJA VIT to the wider population, however, is limited by highly restricted JJA venom (JJAV) extract availability, production cost, and high incidence of adverse events [3–5].

An adjuvant that could facilitate a desirable immune response to JJAV and allow allergen dose reduction would have the potential to minimise adverse effects and reduce cost by reducing venom requirements [6]. A potential candidate adjuvant for VIT is the delta inulin-based adjuvant, Advax™. Advax is based on the plant derived fructan inulin (β-D-[2→1] poly(fructo-furanosyl) α-D-glucose) [7]. Inulin has no immunological activity when in soluble form, but once formulated into delta inulin microparticles of 1–5 μm size, it has potent immune-adjuvant activity [8]. Advax has been shown in preclinical studies to enhance immunogenicity of a broad range of vaccines including influenza, hepatitis B virus (HBV), Japanese encephalitis, SARS coronavirus, HIV, listeria, RSV, and anthrax [9,10]. The safety and efficacy of Advax have been shown in adult humans when formulated with HBV and influenza vaccines [9,11,12]. When combined with honeybee VIT and administered to individuals with honeybee-sting anaphylaxis, Advax was shown to be safe and well tolerated [13], and enhanced the immunogenicity of the honeybee venom with strongly enhanced venom-specific IgG4 responses [14], a potential marker of successful immunotherapy [15].

Understanding the stability of JJA VIT and its aggregation potential with Advax microparticles in a combined formulation is essential as aggregation or decomposition of allergen components in JJAV may affect their allergenic potency [16], which has been found to increase with storage in a previous study [17]. The objective of the current study was therefore to explore the stability and aggregation potential of JJA VIT and Advax in a combined formulation as a prelude to a clinical trial of low-dose JJA VIT with Advax. Although there are currently no animal models of JJAV allergy, it was further necessary to demonstrate using a murine immunogenicity model that co-administration of JJA VIT with Advax was safe and well tolerated and that the inclusion of the Advax adjuvant was able to enhance the immunogenicity of low-dose JJAV.

2. Materials and methods

2.1. Materials

Chemicals, reagents and consumables used in the experiments were as previously described [16]. Chemicals used in the formulation of JJA VIT products were of pharmacopoeial grade; sucrose and polysorbate 80 from Avantor (Centre Valley, PA, USA), benzyl alcohol from PCCA (Houston, TX, USA), sodium chloride 20% solution from AstraZeneca (North Ryde, NSW, Australia), sodium dihydrogen phosphate solution from Phebra (Lane Cove West, NSW, Australia), disodium hydrogen phosphate from SAFC (St. Louis, MO, USA), 0.9% sodium chloride from Pfizer (West Ryde, NSW, Australia), Water for Injection from Pfizer and Baxter (Old Toongabbie, NSW, Australia). Microbiological media and ATCC® strains microorganisms were from Oxoid (Basingstoke, Hampshire, UK). Pooled positive sera (PPS) with high JJAV-specific positive IgE levels was obtained from JJAV allergic individuals with clinically proven history of allergy, as previously described [16].

2.2. Jack Jumper ant venom immunotherapy and Advax adjuvant

JJAV Active Pharmaceutical Ingredient (API) was prepared as previously described [16] and formulated in 22% sucrose solution to produce clinical grade JJA VIT containing 1.1 mg/mL venom proteins. JJAV diluent solution containing 0.9% sodium chloride, phosphate buffer (pH 6.0; 10 mM), 0.05% polysorbate 80 and 0.9% benzyl alcohol was manufactured at the Pharmacy Department, Royal Hobart Hospital as previously described [17]. Advax adjuvant in buffered saline (pH 7.5–8.5) was supplied by Vaxine Pty Ltd (Bedford Park, Adelaide, Australia). The Advax adjuvant was prepared according to current Good Manufacturing Practice [8] and supplied as a sterile suspension containing 50 mg/mL delta inulin microparticles.

2.3. Preparation of JJA VIT/Advax formulations and stability trial conditions

For Advax-free samples, JJAV was diluted to 25 μg/mL using JJAV diluent. Where Advax was included in the formulation, JJA VIT and Advax were first diluted using JJAV diluent to 50 μg/mL and 20 mg/mL, respectively. When the two diluted components were mixed in equal quantities this gave final concentrations of 25 μg/mL and 10 mg/mL, respectively, the planned doses to be used in the future human trial. For allergen potency studies, JJAV was further diluted using JJAV diluent and Advax to obtain final concentrations of 10, 1 and 0.1 μg/mL of venom and 5 mg/mL of Advax. Samples were prepared using aseptic techniques and packaged in U-100 insulin syringes (Terumo, Elkton, Maryland, USA), protected from light and stored at 4 °C and 25 °C for up to 28 days.

2.4. Antigen extraction, efficiency of antigen extraction and control of samples pre-analysis

Baseline samples were processed within 15 min post preparation. All stability samples were collected and processed after 2, 6, 24 and 48 h, and the 25 μg/mL samples were also collected at 3, 7, 14, 21, and 28 days post storage. Except for the particle size analysis, where samples were analysed without antigen extraction, JJAV was extracted from the stability samples by centrifugation. Samples were transferred to Protein LoBind microtubes (Eppendorf AG, Hamburg, Germany) and centrifuged for 5 min at 5000 rpm (2348 × g) and 5 °C (Eppendorf 5424R Microcentrifuge) to pellet the Advax particles. Supernatant was transferred to fresh microtubes and stored at −80 °C until analysis.

2.5. Analysis of JJAV allergen stability

2.5.1. ELISA inhibition

Allergenic potency in 25, 10, 1, and 0.1 μg/mL samples was analysed using JJAV IgE-specific ELISA inhibition assay, as previously described [16]. Briefly, 96-well Amino Immobilizer plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 100 μL of 10 μg/mL JJAV in sodium carbonate (0.05 M, pH 9.6). Inhibition mixtures were prepared by adding between 0.15 and 4 ng of JJAV to 25 μL PPS and diluting to 100 μL with phosphate buffered saline (0.01 M, pH = 7.2) containing 0.05% polysorbate 20 (PBS-T) and 0.5% bovine serum albumin (BSA). JJAV in the inhibition mixtures was either baseline or stability samples as described in Section 2.4. A positive control was prepared by diluting PPS 1:4 (v/v) in PBS-T with 0.5% BSA and negative control consisted of PBS-T with 0.5% BSA and incubated overnight at 4 °C. Subsequently, venom coating solution was discarded and the plate was washed three times with PBS-T. The venom-coated plate was incubated with 100 μL of inhibition mixtures, positive control and negative control for 60 min at room temperature (RT). The plate was washed three times with

PBS-T, and 100 µL of biotinylated mouse monoclonal anti-human IgE antibody (1:2000 (v/v) dilution in PBS-T, clone HP6029, Life Technologies, Carlsbad, CA, USA) was added to each well and incubated in the dark for 60 min at RT. The plate was washed three times with PBS-T, and then 100 µL of streptavidin–horseradish peroxidase (1:1000 (v/v) dilution in PBS-T, BD Pharmingen, San Diego, CA, USA) was added to each well and the plate was incubated in the dark for 20 min at RT. The plate was washed three times with PBS-T, and then 100 µL of 1-Step Ultra TMB-ELISA (Thermo Fisher Scientific, Rockford, IL, USA) was added to each well and the plate was incubated in the dark for 30 min at RT. Colour development was stopped by adding 100 µL of 2 M sulphuric acid and absorbance was read at 450 nm utilizing an ELISA plate reader (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA). Samples were run in triplicates, and the mean and standard deviation calculated. The percentage inhibition for each sample was calculated using the following equation:

$$\text{Inhibition of IgE binding (\%)} = \frac{\text{Absorption of positive control} - \text{Absorption of inhibited sample}}{\text{Absorption of positive control}}$$

Allergenic potency of JJAV was measured as the amount of venom in solution that was required to inhibit 50% of IgE binding to the JJAV coated onto the plate. The stability of venom samples were calculated as a percentage relative to baseline samples.

2.5.2. SDS-PAGE and SDS-PAGE resolved JJAV immunoblot

The 25 µg/mL samples were concentrated to 0.5 mg/mL using a centrifugal filter device (Amicon Ultra-0.5 with 3 kDa nominal molecular weight limit, Merck Millipore, Darmstadt, Germany), after which they were mixed with SDS sample buffer and analysed by SDS-PAGE or SDS-PAGE immunoblot assay as previously described [16]. PPS was used in the SDS-PAGE immunoblot assay.

2.5.3. Ultra performance liquid chromatography

To assess Myr p allergen peptide changes, the 25 µg/mL venom samples were analysed by UPLC-UV using an Acquity H-series UPLC (Waters Corporation, Milford, Massachusetts, USA) coupled to an Acquity Photo Diode Array (PDA) detector. An Acquity UPLC BEH C18 column (2.1 x 100 mm x 1.7 µm particles) held at 40 °C was used. The mobile phase flow rate was 0.35 mL/min and the solvent system was water:acetonitrile:1% trifluoroacetic acid in water (80:12:8, v/v/v) to (24:68:8, v/v/v) in a linear gradient over 12 min before re-equilibration for 3 min to initial conditions. Triplicate injections of 40 µL were made and data for quantitative measurements of peak areas corresponding to the Myr p 1–3 allergen peptides were extracted at 220 nm. Under these conditions Myr p 2 eluted at 7.22 min, Myr p 3 at 7.52 min, and Myr p 1 at 8.39 min (Fig S1). Data were analysed using Waters MassLynx and TargetLynx software. The mean peak area for each allergen peptide was calculated and the analysis of remaining concentration of the allergen peptides was calculated as a percentage relative to baseline samples.

2.6. Analysis of JJAV diluent stability

2.6.1. Measurement of pH

Sample(s) pH was measured using a calibrated pH meter (Hanna Instrument, Woonsocket, RI, USA).

2.6.2. Benzyl alcohol determination

Benzyl alcohol concentration was determined by UPLC-UV using the instrument and column described above. The mobile phase flow rate was 0.35 mL/min and the solvent system was an isocratic mixture of 1% acetic acid in water:acetonitrile (70:30, v/v). Quadruple injections of 20 µL were made and data for quantitative measurements were extracted at 270 nm to maintain on scale signals. Benzyl alcohol eluted at 1.3 min and the peak area was determined using Waters TargetLynx software. Where benzyl alcohol-free Advax was added, the concentration of benzyl alcohol in the JJAV diluent was reduced by 19%, consistent with dilutional effect.

2.7. Analysis of advax stability

Particle size of Advax (10 mg/mL) in the presence of JJAV (25 µg/mL) in diluent solution was determined by dynamic light scattering (DLS) using a Zetasizer Nano S (Malvern Instruments Ltd., Worcestershire, UK). Samples were mixed by vortexing to form a uniform suspension and transferred to UVette plastic cuvettes (Eppendorf AG). Sample compartment temperature was adjusted to 25 °C before measurements were taken. Measurements were performed in triplicate and the average particle size (Z-Average size) and particle size distribution (via Polydispersity Index; PdI) were obtained [18].

2.8. Microbiological analysis

2.8.1. Endotoxin content

Samples were analysed for endotoxin contents using Limulus Amebocyte Lysate QCL-1000 chromogenic assay (Lonza, Walkersville, MD, USA) as previously described [16].

2.8.2. Antimicrobial efficacy test

The antimicrobial efficacy of benzyl alcohol in JJAV diluent was evaluated using the United States Pharmacopoeia Antimicrobial Effectiveness Test (AET) [19]. The following challenge organisms were used: *Candida albicans* (ATCC 10231), *Escherichia coli* (ATCC 25922), and *Staphylococcus aureus* (ATCC 25923). Bacterial stock cultures were grown for 20–24 hours in Tryptone Soya Broth and yeast stock culture was grown for 44–48 hours in Sabaroud Dextrose Broth. Microorganisms were harvested by centrifugation, washed, resuspended, and diluted in 0.9% normal saline to obtain a microbial count of approximately 1×10^8 colony forming units per mL (CFU/mL). Microbial suspensions were used within 30 min of harvest. For each challenge organism, AET was conducted in five replicates. Test samples consisting of 400 µL of JJAV diluent containing either JJAV (25 µg/mL) alone or with Advax (10 mg/mL), were inoculated with the microbial suspension at 1.0% (v/v) to yield a final concentration between 1×10^5 and 1×10^6 CFU/mL and incubated at 23 °C for up to 28 days. Aliquots (100 µL) were taken from each sample at days 7, 14 and 28, and the number of CFUs in each sample was determined using plate-count method. Positive control samples (400 µL of 0.9% normal saline) were inoculated with the microbial suspension at 1.0% (v/v) to yield a final concentration between 1×10^5 and 1×10^6 CFU/mL. A 100 µL aliquot was taken from each control sample at baseline and the number of CFUs was determined using plate-count method. Negative control samples were growth media (from the same batches) devoid of any manipulation.

2.9. Murine immunogenicity studies

To assess the potential of Advax to provide JJAV antigen-sparing, female BALB/c mice, 6–8 week old were immunized 4 times intramuscularly at 2-week intervals with JJAV 2 µg (Royal Hobart Hospital, Tasmania, Australia) alone or formulated with Advax 1 mg (Vaxine Pty Ltd, Adelaide, Australia) versus JJAV 10 µg alone in 50 µL total injection volume. Blood samples were collected 2 weeks after the last immunization for measurement of JJAV-specific IgG responses by ELISA.

Table 1
Acceptance criteria for physicochemical and microbiological stability of JJA VIT, JJAV diluent and Advax adjuvant.

Characteristic	Assay	Predetermined criteria
JJAV allergens stability		
Allergenic potency	ELISA inhibition	50 - 150% relative to baseline
Individual allergen concentration	UPLC-UV	75 - 150% relative to baseline
Protein profile	SDS-PAGE	Identical to baseline
Allergen profile	SDS-PAGE immunoblot	Identical to baseline
JJAV diluent stability		
Potential of hydrogen	pH	5.9 - 6.3
Benzyl alcohol concentration	UPLC-UV	> 90% relative to baseline
Advax adjuvant stability		
Particle diameter	Dynamic Light Scattering	1 - 7 μm
Microbiological purity		
Endotoxin content	Limulus Amebocyte Lysate QCL-1000	< 50 EU/ml
Preservative efficacy	Antimicrobial Efficacy Test	1×10^5 - 1×10^6 CFU/plate on baseline; ≥ 1 log reduction relative to baseline on day 7; ≥ 3 log reduction relative to baseline on day 14; no increase on day 28 relative to day 14 count

2.9.1. JJAV-specific antibody ELISA

Mouse JJAV-specific IgG, IgG1 and IgG2a antibodies were determined by ELISA, as previously described, with minor modifications [20]. Briefly, JJAV (1 μg/mL) in coating buffer (pH 5.0; 50 mM MES + 25 mM HEPES in PBS) was absorbed to 96 well plates, blocked with 0.2% Casein, then 100 μL of 1:200 dilution of sera was incubated for 2 h at RT, followed by washing then incubation with biotinylated anti-mouse IgG, IgG1 and IgG2a antibodies (Abcam, Cambridge, MA, USA) mixed with HRP-conjugated Streptavidin (BD Biosciences, Franklin Lakes, NJ, USA) for 1 h. Washed wells were then incubated with TMB substrate (KPL, Gaithersburg, MD, USA) for 10 min before the reaction was stopped with 1 M phosphoric acid and optical density measured at 450 nm ($OD_{450\,nm}$) using a VersaMax plate reader and analysed using SoftMax Pro software (Molecular Devices, Sunnyvale, CA, USA). Average $OD_{450\,nm}$ values obtained from negative control wells were subtracted.

2.10. Statistical and data analysis

Statistical analysis was performed using GraphPad Prism 5.03 and CurveExpert Basic 1.4. Statistical analyses, including descriptive statistics, *t*-test and ANOVA with Bonferroni's post-hoc test were used as appropriate, and $p < 0.05$ was considered significant. Image analysis was performed using ImageJ 1.46 r (Wayne Rasband, National Institutes of Health). For the noninferiority analysis, we performed an independent *t*-test on the log transformed IgG data, and assessed whether the upper limit of the 95% confidence interval for the difference between treatments (low dose adjuvanted JJAV treatment minus standard high dose JJAV alone treatment) was below a predefined margin of non-inferiority of 0.5.

2.11. Stability criteria

Table I describes the acceptance criteria for the physicochemical and microbiological stability of JJA VIT components and Advax adjuvant.

3. Results

3.1. The effect of Advax on the allergenic potency of JJAV

The allergenic potency of JJAV at baseline was not significantly affected by the presence of Advax in the formulation ($p = 0.06–0.99$; Supplementary Table 1). The overall allergenic potency of JJAV with and without Advax was acceptable for at least 2 days when stored at 4 and 25 °C, with relative allergenic potencies between 102.4–139.0% and 97.5–144.9% for all stability samples stored at 4 °C with and without Advax respectively, and between 100.2–134.3% and 94.3–126.0% for all stability samples stored at 25 °C with and without Advax respectively (Figs. 1 and 2). A trend for a progressive increase in allergenic potency was observed in the 25 μg/mL JJAV stability samples, which continued to increase beyond the acceptable limits of 50–150% after 14 days of storage at either 4 or 25 °C, particularly for the stability samples with Advax (Fig. 1).

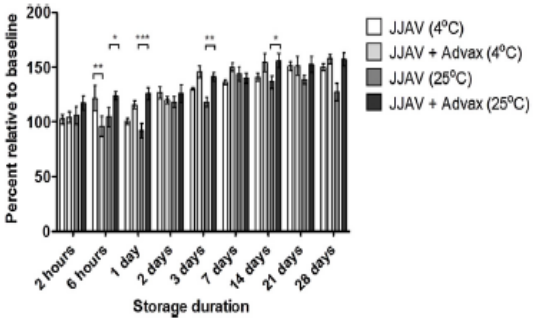

Fig. 1. Effects of Advax (10 mg/mL) and storage temperature (4 °C or 25 °C) on the allergenic potency of JJAV (25 μg/mL) stored for up to 28 days. Analysis of allergenic potency was calculated as a percentage relative to baseline samples. Each sample was analysed in triplicate and presented as mean and SD. Asterisks designate significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.2. The effects of Advax on the stability of JJAV allergens

Myr p 1 was the least stable of the 3 allergens that were quantified via UPLC-UV, and as such, stability was limited by the stability of Myr p 1 (Fig S1). Therefore, in the presence of Advax, the JJAV was stable for up to 7 and 3 days when stored at 4 °C and 25 °C, respectively (Fig. 3). No significant differences were found between 'with' and 'without' Advax for either baseline samples (Supplementary Table 2) or for all but one of the stability pairs (Fig. 3). SDS-PAGE results showed that a protein band at approximately 23 kDa disappeared slowly throughout the storage period and appeared to be replaced with new bands at approximately 17, 19 and 21 kDa (Fig S2). The changes occurred regardless of sample treatments and were more obvious with prolonged storage. A similar pattern was also observed on the SDS-PAGE immunoblot results, whereby an IgE-binding band at 23 kDa slowly disappeared and was replaced with a new IgE-binding band at 19 kDa (Fig S3). The changes occurred regardless of Advax, and were most obvious in the stability samples stored at 25 °C for 24 h.

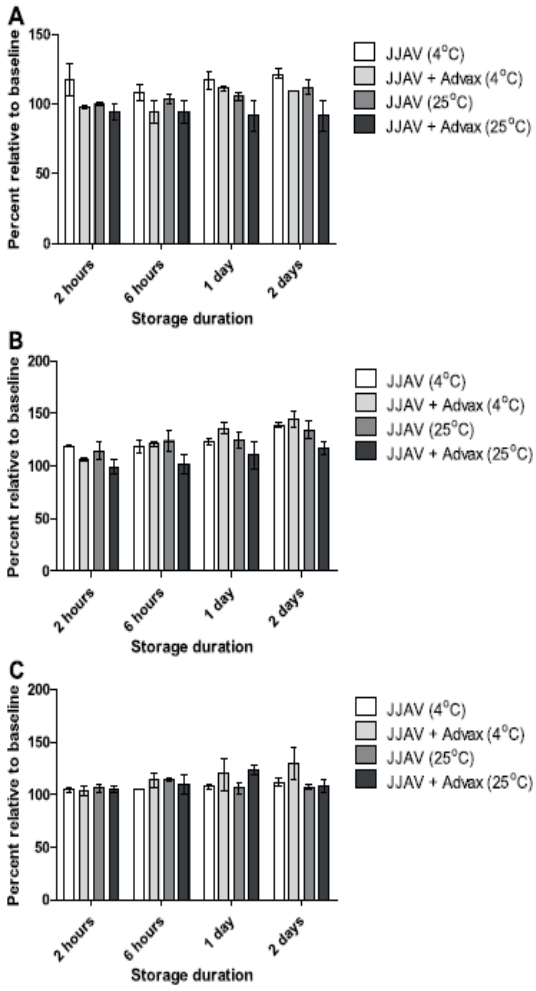

Fig. 2. Effects of Advax (5 mg/mL) and storage temperature (4 °C or 25 °C) on the allergenic potency of JJAV at (A) 0.1 µg/mL, (B) 1 µg/mL, and (C) 10 µg/mL stored for up to 2 days. Analysis was calculated as a percentage relative to baseline samples. Each sample was analysed in triplicate and presented as mean and SD.

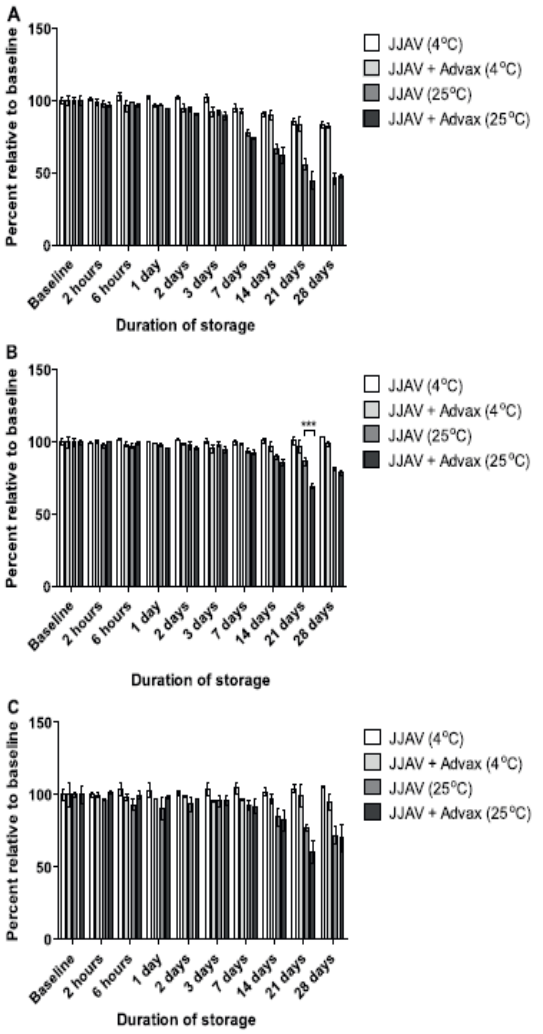

Fig. 3. Effects of Advax (10 mg/mL) and storage temperature (4 °C or 25 °C) on JJAV allergen peptides (A) Myr p 1, (B) Myr p 2, and (C) Myr p 3 in formulations of JJAV (25 µg/mL) stored for up to 28 days. Analysis of remaining concentration of Myr p allergen peptides was calculated as a percentage relative to baseline samples. Each sample was analysed in triplicate and presented as mean and SD. Asterisks designate significant differences (*** $p < 0.001$).

3.3. The effects of Advax on JJAV diluent stability

The addition of Advax did not cause a substantial change in the pH of JJAV diluent (Fig S4), and storage temperature had a minimal effect on the stability of benzyl alcohol for the period studied (Fig S5).

3.4. Size distribution of advax microparticles formulated with JJA VIT

Advax delta inulin microparticles had a mean hydrodynamic diameter of 1.6 µm at baseline (Fig. 4A), and this did not change after storage at 4 and 25 °C for up to 21 and 7 days respectively, but a two-fold increase in diameter was observed at the 28 day study endpoint at both 4 and 25 °C. The polydispersity index (PdI) of Advax was approximately 0.6 at baseline and did not change substantially between day 1 and 21 of the study period, although there were noticeable fluctuations over time, particularly in the sample stored at 25 °C, and the PdI increased to 1.0 at day 28 of study period at both 4 and 25 °C (Fig. 4B).

3.5. Antimicrobial activity of JJA VIT formulated with Advax

The batches of JJA VIT, JJAV diluents, and Advax were virtually free of endotoxins (Supplementary Table 3), and sterility testing of JJA VIT and JJAV diluents further supported the aseptic nature of the combined formulation (data not shown). All samples including JJAV diluent containing only JJAV and samples where Advax was added met the Pharmacopoeial requirements for antimicrobial activity at each sampling time point (Supplementary Table 4).

3.6. Effect of Advax adjuvant on immunogenicity and tolerability of JJA VIT

A murine immunogenicity model was used to test the hypothesis that Advax adjuvant could be used for JJAV dose sparing and to confirm that it had no negative effects on safety and tolerability. Female BALB/c mice, 6–8 week of age (n = 5/group), were immunized 4 times intramuscularly at 2-week intervals with JJAV 2 µg alone or combined with 1 mg Advax adjuvant versus JJAV 10 µg

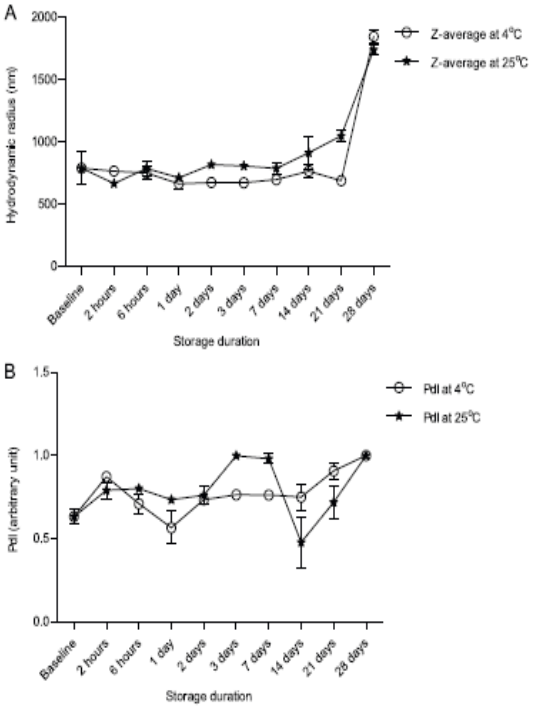

Fig. 4. Particle size distribution of Advax (10 mg/mL) as a function of (A) Z-average and (B) polydispersity index (PdI) when combined with JJAV (25 μg/mL) and stored for up to 28 days at either 4 °C or 25 °C. Analysis was performed using diffraction light scattering technique. Each sample was analysed in triplicate and presented as mean and SD.

alone, with the aim to assess the non-inferiority of the JJAV specific IgG responses in the JJAV 2 μg + Advax low dose group when compared to the JJAV 10 μg high dose group. Blood samples were collected 2 weeks after the last immunization for measurement of JJAV-specific total IgG and IgG subtypes by ELISA. While only low or undetectable levels of JJAV-specific IgG were seen in animals immunised with 10 μg of JJAV alone, all animals in the low dose JJAV (2 μg) + Advax arm had detectable JJAV-specific IgG. A formal non-inferiority analysis performed using pre-specified limits, confirmed the low dose JJAV + Advax arm was statistically non-inferior to the high dose JJAV alone group ($p = 0.976$). A subsequent test of superiority confirmed that the JJAV-specific IgG responses in the low dose JJAV + Advax arm were superior ($p = 0.048$) to those in the high dose alone arm (Fig. 5). Hence, Advax was confirmed to provide at least 5-fold JJAV dose-sparing. Analysis of IgG isotypes confirmed that the predominant effect of Advax adjuvant was predominantly through significant enhancement of specific IgG1 rather than IgG2a. No JJAV-specific IgE was detected in the sera of the immunized mice and no local (swelling, redness, hair loss) or systemic (weight loss, fever, inactivity, loss of grooming) adverse effects were observed.

4. Discussion

Preclinical studies of novel adjuvanted allergen-specific immunotherapy (AIT) formulations are generally required by regulatory authorities prior to initiation of clinical trials. Such studies should demonstrate physicochemical and microbiological compatibility of the adjuvant with the antigenic components contained within the vaccines [21], together with immunogenicity data to justify the inclusion of the adjuvant in the vaccine. Although many human trials have been conducted on Advax adjuvant in combination with various infectious disease vaccines and one study of Advax with honeybee VIT [8,13,14], no information was previously available on the formulation, stability or immunogenicity of JJAV antigens in the presence of Advax.

In the current study, we performed stability study of low-dose JJA VIT with and without Advax adjuvant, pre-packaged in plastic syringes thereby confirming that the JJA VIT and Advax components were compatible with each other, and minimal aggregation of delta inulin microparticles was observed in the period studied. Based on the data obtained, in particular the allergenic potency studies, low-dose JJA VIT formulated with Advax is both physicochemically and microbiologically stable for at least 2 days when stored at 4 °C and 25 °C in plastic syringes. This will allow low-dose JJA VIT, formulated with or without Advax, to be prepared at a central pharmacy location in advance of clinical trial use and then transported to the site of patient administration, removing the need to prepare the vaccine at the patient's bedside and may reduce the likelihood of errors in dosing occurring [22].

The JJAV stability results obtained in the current study are consistent with a previous report [17], although the conditions employed were somewhat different. In particular, the current study used U-100 plastic insulin syringes to store the samples and a lower concentration of JJAV was used than in the previous study. Measurement of total allergenic activity as determined by ELISA inhibition assay is required by regulatory bodies for the standard-

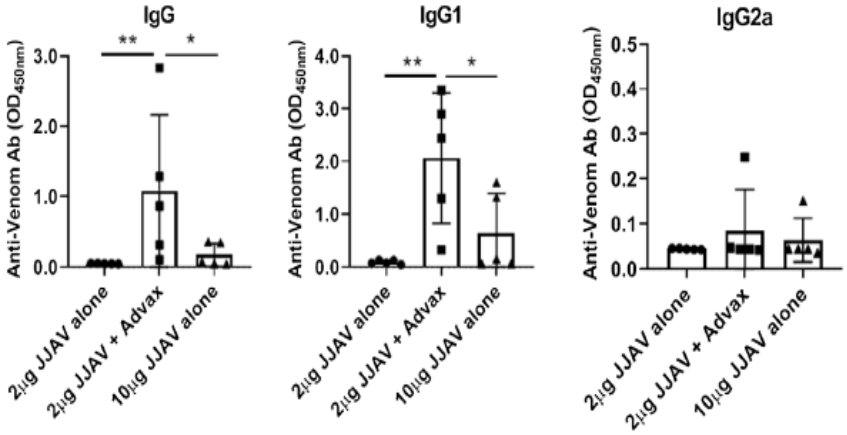

Fig. 5. Antigen-sparing effect of Advax adjuvant on JJAV-specific IgG responses. Female BALB/c mice, 6–8 week old (n = 5/group) were immunized 4 times i.m. at 2-week intervals with JJAV 2 μg alone or with Advax 1 mg compared to a 5-fold higher dose of JJAV 10 μg alone, all in 50 μL total volume. Blood samples were collected 2 weeks after the last immunization and JJAV-specific IgG, IgG1 and IgG2a measured by ELISA (* $p < 0.05$, ** $p < 0.01$).

ization and batch control of AIT products [23,24]. The slight increase in JJAV allergenic activity with storage is entirely consistent with previous findings and this has been proposed to be due to conformational changes in the JJAV proteins in the presence of polysorbate 80 in the JJAV diluent [17]. Despite ELISA inhibition data indicating that the vast majority of allergenic activity was preserved, it does not exclude the possibility that one or more "minor" allergens might have degraded. This perhaps is better monitored using the immunoblot assay where we observed the shift down in the 22–23 kDa bands, recently found to be Phospholipase A_2 enzyme [1], to a band at approximately 19 kDa (Fig S2). This pattern of allergen degradation is consistent with prior findings and it was not affected by the inclusion of Advax in the formulation. Wanandy et al. recently reported the adsorption of Myr p 1 and Myr p 3 allergens when JJAV API grade products were allowed to contact rubber stoppers for more than 24 h [16]. Even though the low-dose JJAV in the current study was stored in plastic syringes with rubber plungers, negligible adsorption was identified. This difference might be due to the inclusion of polysorbate 80 as a surface active agent in the current samples, or different adsorption capacity and surface area of the rubbers.

JJAV solutions above 25 μg/mL that are used for immunotherapy are intended for multi-dose use. The addition of benzyl alcohol as an antimicrobial preservative in the JJAV diluent is essential to reduce the risk of bacterial contamination [25,26]. The insignificant change in the pH of the JJAV diluent in the presence of Advax was important as the solubility and optimal antimicrobial activity of benzyl alcohol is considerably reduced above pH 7 [17,27]. When used as an antimicrobial preservative, benzyl alcohol is commonly added to parenteral preparations at a concentration between 0.75–5% [27,28]. A 19% reduction of benzyl alcohol concentration in JJAV diluent due to dilutional effects from the addition of Advax reduced the concentration of benzyl alcohol to below this usual range and could therefore have affected its effectiveness as a preservative. However, the AET experiments confirmed that the antimicrobial efficacy of the combined formulation against challenged organisms was maintained throughout the study period. It is possible that this was due, at least in part, to the inherent antimicrobial activities of JJAV [29], which provided additive or synergistic effects to the preservative activity of benzyl alcohol. If this was the case, since low concentrations of venom proteins are used during the induction phase of a VIT, a greater reliance on the antimicrobial efficacy of benzyl alcohol is required during this phase.

A PdI of < 0.7 suggests a monodisperse preparation [18], and the results from these studies showed that Advax had a relatively monodisperse particle size distribution at the start of the study as indicated by the PdI of 0.6. The increase in average particle size and PdI observed at the end of study period may be caused by formation of delta inulin aggregates. However, the absence of multimodal peaks in all samples tested throughout the study period suggests that aggregation, if present, is limited under the study conditions employed and importantly the size of these particles was within the accepted range for stability (Table 1). Advax has been reported to have minimal protein adsorptive capacity [30], and similarly we found no evidence that Advax adsorbs JJAV allergens as there was minimal difference in the SDS-PAGE and UPLC-UV analysis of venom components between samples formulated with and without Advax.

Notably, the murine immunogenicity data supported the hypothesis that Advax adjuvant can provide significant antigen-sparing for JJA VIT, inducing significantly higher serum JJAV-specific IgG levels in the mice that received the adjuvanted venom formulation than a five times higher dose of JJAV alone. Whilst there is currently no animal model of JJAV allergy, and hence the effects of Advax on inhibition of JJAV IgE during VIT or its ability to induce blocking IgG4 antibodies is unable to be assessed outside of human studies, the ability of Advax to enhance venom-specific IgG responses when compared to immunisation with venom alone has served as a useful predictive marker of favourable human responses in previous human studies of Advax-adjuvanted honeybee VIT, where inclusion of Advax led to an earlier and higher rise of venom-specific total IgG and IgG4 with potential blocking activity [13,14]. Hence the current results support the rationale for a planned human trial of Advax combined with a reduced dose of JJAV (25 μg in maintenance phase) as compared to the currently clinically proven 100 μg maintenance JJAV dose. Notably, no safety or reactogenicity issues were identified with the Advax-adjuvanted low dose JJAV formulation in the current study.

5. Conclusion

Advax adjuvant provided at least 5-fold JJAV antigen sparing in murine immunogenicity studies, with no observed issues of reactogenicity or safety. No detrimental effects of Advax on JJAV stability were found, supporting use of this combined formulation as a JJAV-sparing strategy in planned human trials. Plans are in place to commence a human clinical trial to assess the ability of Advax to reduce the required maintenance dose of JJAV from 100 to 25 μg per dose, which if successful would result in a 4-fold higher number of subjects able to be treated with JJA VIT with current supplies, while also significantly reducing the potential cost of therapy, which largely reflects JJAV costs.

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Contributors

RJH, SGAB, NP and MDW conceived the study. TW, MDW and NP designed the study methodologies. TW, NWD, HER and YH-O performed the experiments. TW, NWD, NP, RW and MDW analysed the data. TW prepared the manuscript. All authors participated in its revisions and have approved the final form.

Conflict of interest

NP and YH-O are associates of Vaxine Pty Ltd which owns the Advax™ adjuvant platform. TW, HER, SGAB, and MDW are either employee or consultants to the Tasmanian Health Service, the organization owning the intellectual property over JJA VIT. All other authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2019.04.017.

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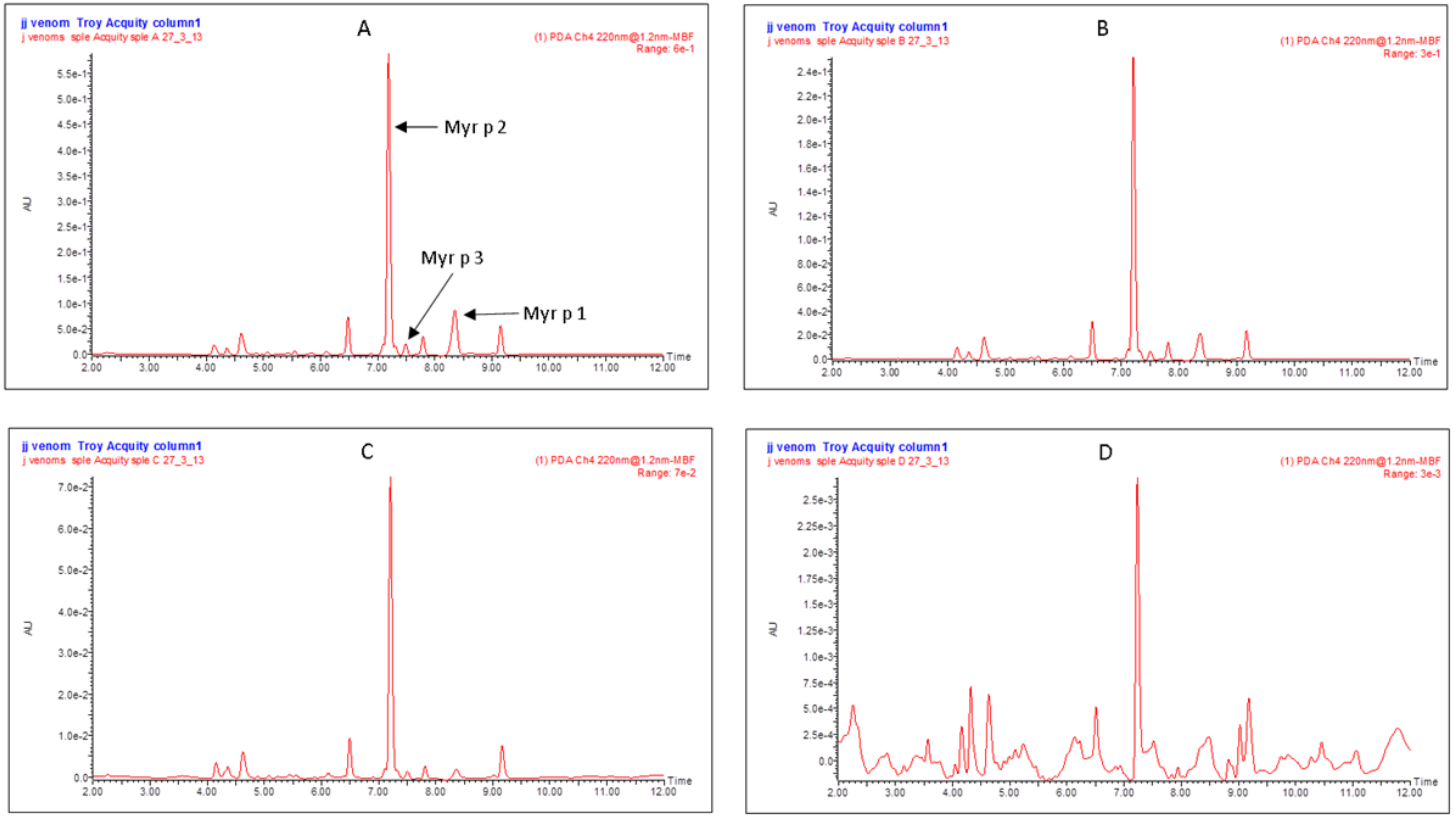
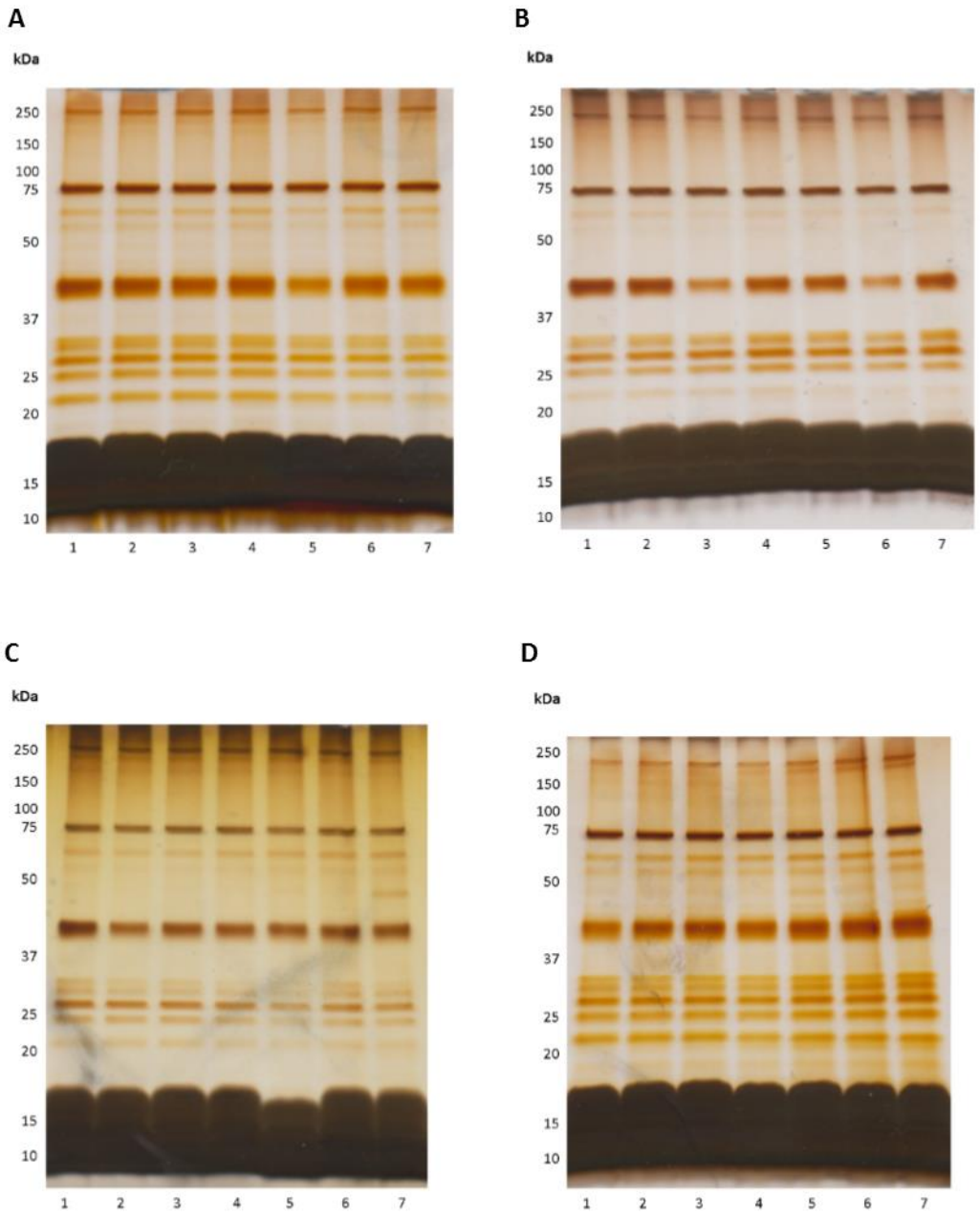
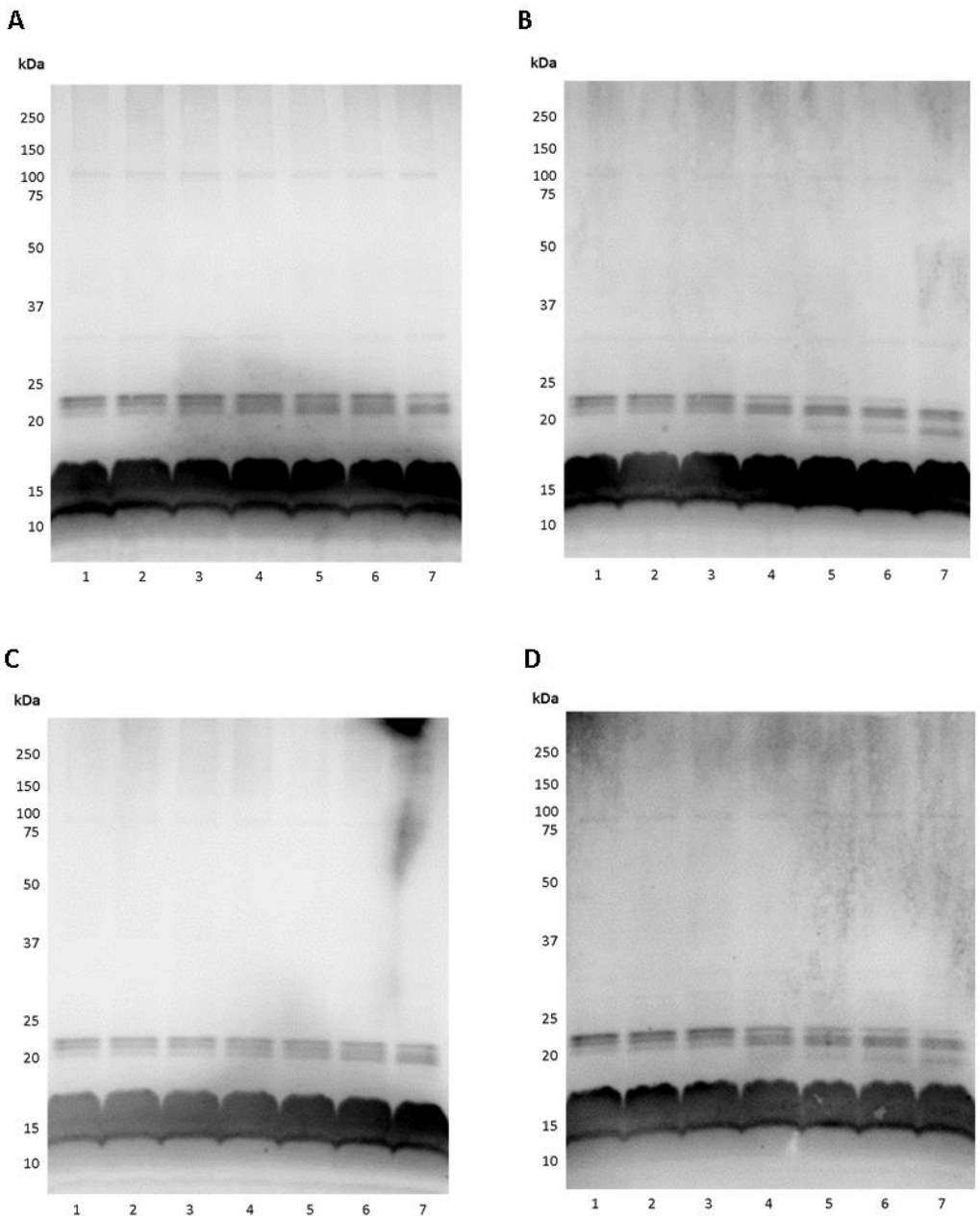
6.3. Supporting information



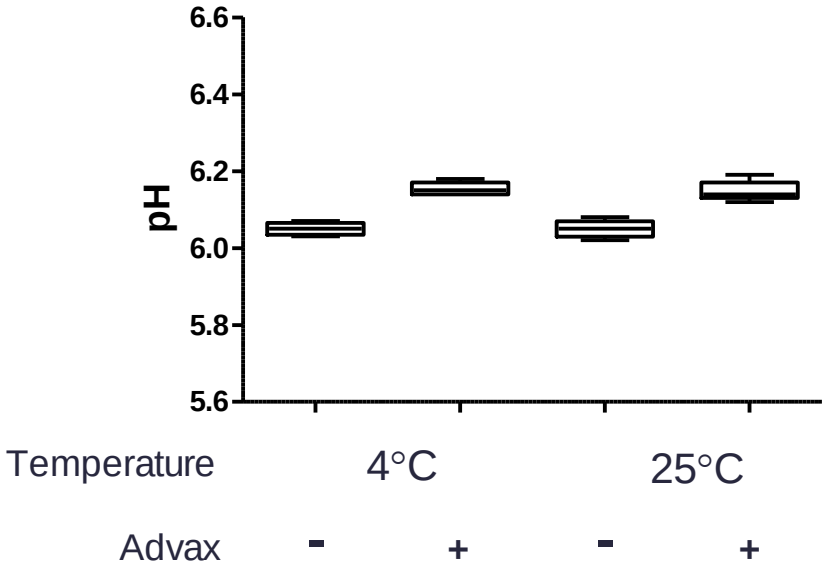
Supplementary Figure 1. Representative chromatograms of JJAV samples containing (A) 100 µg/mL, (B) 50 µg/mL, (C) 25 µg/mL, and (D) 10 µg/mL of venom protein. Samples were analysed using UPLC-UV coupled with Waters Acquity BEH C18 column and Photo Diode Array (PDA) detector. Peaks corresponding to the Myr p 1, Myr p 2, and Myr p 3 allergenic peptides are shown in panel (A).



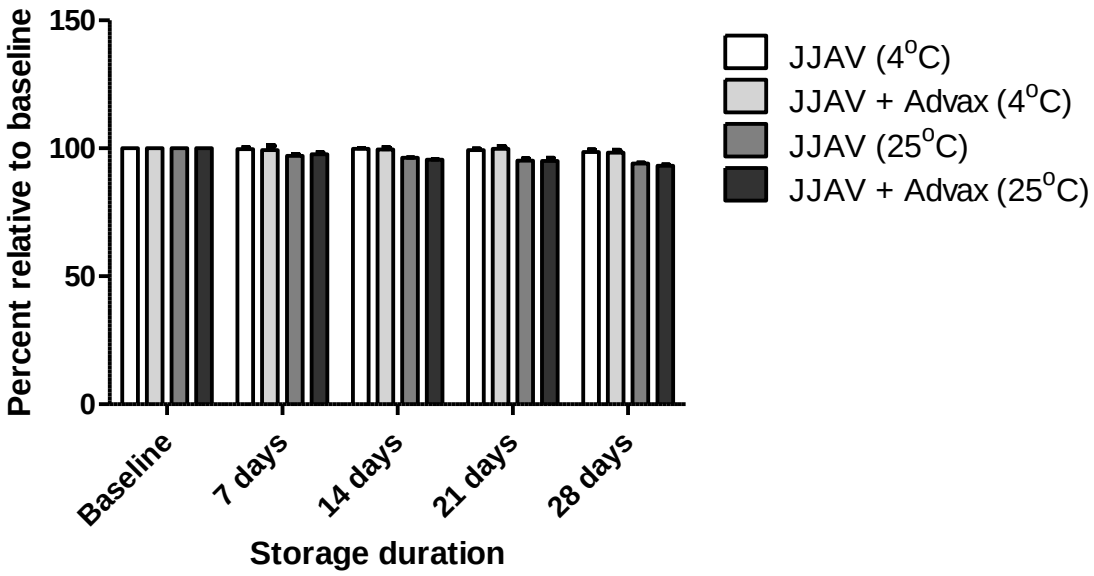
Supplementary Figure 2. SDS-PAGE analysis of venom components in JJAV (25 µg/mL) stored at 4^{o}C (A) without Advax and (C) with Advax (10 mg/mL), or at 25^{o}C (B) without Advax and (D) with Advax (10 mg/mL) for up to 7 days. Lanes: 1. Baseline, 2. After 2 hours, 3. After 6 hours, 4. After 1 day, 5. After 2 days, 6. After 3 days, and 7. After 7 days of storage.



Supplementary Figure 3. SDS-PAGE Immunoblot analysis of IgE binding venom components in JJAV (25 µg/mL) stored at 4^{o}C (A) without Advax and (C) with Advax (10 mg/mL), or 25^{o}C (B) without Advax and (D) with Advax (10 mg/mL) for up to 7 days. Lanes: 1. Baseline, 2. After 2 hours, 3. After 6 hours, 4. After 1 day, 5. After 2 days, 6. After 3 days, and 7. After 7 days of storage.



Supplementary Figure 4. Effects of Advax (10 mg/mL) and storage temperature (4^{o}C or 25^{o}C) on the buffering capacity of phosphate buffered saline in JJAV diluent used in the formulation of JJAV (25 µg/mL) with or without Advax. Analysis of pH was taken from each samples at defined time points for a period of 28 days and presented as mean and SD.



Supplementary Figure 5. Effects of Advax (10 mg/mL) and storage temperature (4°C or 25°C) on benzyl alcohol concentration in JJAV diluent used in the formulation of JJAV (25 µg/mL) with or without Advax. Benzyl alcohol concentration in the samples was quantified against an analytical standard using UPLC-UV. Analysis was calculated as a percentage relative to baseline samples. Quadruple analysis was performed on each sample and presented as mean and SD.

Supplementary Table 1. Allergenic potency of various JJAV concentrations with or without Advax at baseline as determined by JJAV-specific IgE ELISA Inhibition assay

JJAV Concentration	Storage Condition	Advax adjuvant	Allergenic Potency as 50% Inhibition (Mean ± SD; nanogram)	P value
25 µg/mL	4°C	–	0.349 ± 0.072	0.60
		+	0.385 ± 0.081	
	25°C	–	0.501 ± 0.061	0.43
		+	0.424 ± 0.138	
10 µg/mL	4°C	–	0.301 ± 0.002	0.99
		+	0.304 ± 0.230	
	25°C	–	0.276 ± 0.065	0.66
		+	0.335 ± 0.204	
1 µg/mL	4°C	–	0.648 ± 0.316	0.35
		+	0.441 ± 0.113	
	25°C	–	0.480 ± 0.230	0.27
		+	0.291 ± 0.114	
0.1 µg/mL	4°C	–	0.297 ± 0.007	0.06
		+	0.628 ± 0.118	
	25°C	–	0.224 ± 0.001	0.10
		+	0.461 ± 0.116	

P values were calculated using t-test; $p < 0.05$ was considered significant

Supplementary Table 2. Concentration of Myr p allergens at baseline in formulations containing JJAV (25 µg/mL) with or without Advax as determined by UPLC-UV

Storage Condition	Advax adjuvant	Allergen	AUC at Baseline (Mean ± SD)
4°C	–	Myr p 1	3039.22 ± 84.95
		Myr p 2	9421.88 ± 322.82
		Myr p 3	378.76 ± 23.26
	+	Myr p 1	3151.46 ± 161.53
		Myr p 2	9730 ± 541.86
		Myr p 3	392 ± 43.71
25°C	–	Myr p 1	3249.23 ± 102.43
		Myr p 2	9757.38 ± 412.2
		Myr p 3	415.93 ± 11.21
	+	Myr p 1	3179.17 ± 131.13
		Myr p 2	9673.43 ± 257.06
		Myr p 3	412.68 ± 33.95

Supplementary Table 3. Endotoxin level in multiple batches of JJA VIT, JJAV diluents and Advax adjuvant used in the study

Sample	Number of batches	Mean ± SD (EU/mL)
JJA VIT	5	0.028 ± 0.001
JJAV diluent	5	0.022 ± 0.001
Advax adjuvant	3	1.534 ± 0.020

Supplementary Table 4. Antimicrobial efficacy of benzyl alcohol in JJAV diluent used in the formulation of JJAV (25 µg/mL) with or without Advax (10 mg/mL)

Sampling time	Sample	Observed Log reduction (Mean of 5 plates)			Acceptance criterion
		***E. coli* ATCC 25922**	***S. aureus* ATCC 25923**	***C. albicans* ATCC 10231**	
Baseline	0.9% NaCl Control	$1 \pm 1 \times 10^6$ CFU/plate	$4 \pm 1 \times 10^5$ CFU/plate	$3 \pm 3 \times 10^5$ CFU/plate	1×10^5 to 1×10^6 CFU/plate on initial count
Day 7	JJAV	6 Log reduction on both sample types	5 Log reduction	5 Log reduction on both sample types	≥ 1 log reduction from the initial count
	JJAV + Advax		3 Log reduction		
Day 14	JJAV	6 Log reduction on both sample types	5 Log reduction on both sample types	5 Log reduction on both sample types	≥ 3 log reduction from the initial count
	JJAV + Advax				
Day 28	JJAV	6 Log reduction on both sample types	5 Log reduction on both sample types	5 Log reduction on both sample types	No increase from the 14 day count
	JJAV + Advax				

Part III: SUMMARY

Chapter 7: General discussion

7.1. Optimising the delivery of Jack Jumper ant Venom Immunotherapy

I performed the first published study to assess the consistency of Hymenoptera venom allergen extracts and the impact of environmental factors on extract quality (see Chapter 4 for details of this research). My analysis of the 17 batches of purified *M. pilosula* venom extracts in the form of Active Pharmaceutical Ingredients found good batch-to-batch consistency. This finding reinforces our manufacturing requirements for pooling venom sacs obtained from different collection times and locations. In this study, I also observed that the relative amounts of Myr p 3 (Pilosulin 4.1) allergens and endotoxin concentration are two intrinsic factors that may affect the total allergenic potency in *M. pilosula* venom extracts. In addition, prolonged storage above room temperature and contact with rubber stoppers are two environmental factors that impact the amounts of Myr p peptides in venom extracts. Furthermore, prolonged exposure of venom extracts to temperatures above 40^{o}C affects the protein >20 kDa with IgE-binding activity. These results strongly support our current protocol to store collected ants, dissected venom sacs and venom extracts at ultra-low temperature (–20^{o}C or below). This requirement will control bio-burden level (and hence endotoxin concentration) and the preservation of allergenic components and allergenic potency in venom extracts. A protocol for storing vials containing *M. pilosula* venom extracts in an upright position will limit the adsorption of allergenic components into rubber stoppers. These requirements are important to maintain the diagnostic utility of intradermal testing reagents and efficacy of Allergen Immunotherapy products (348), and the data generated from this study have been collated into published guidelines for the storage, handling and transport of *M. pilosula* venom extracts for practicing clinicians (349).

Using serum samples from patients with clinically diagnosed allergy to *M. pilosula* venom, I confirmed previous observations (11, 174), which reported that a number of high molecular weight proteins >20 kDa in *M. pilosula* venom are capable of binding to IgE. Importantly, my analysis showed that some of these proteins are recognized by the immune system in a considerable number of patients who are allergic to *M. pilosula* venom (see Chapter 5 for details of this research). My observation that these proteins are susceptible to

elevated temperature, which potentially affects quality, safety and efficacy of our Allergen Immunotherapy products and thus patients care, further justifies the need to identify these venom components. Separation of proteins in native venom from the more abundant Myr p peptides by Size Exclusion Chromatography followed by SDS-PAGE, immunoblot and tandem mass spectrometry enabled me to establish the identity of the venom proteins with and without IgE-binding properties. Classical (e.g. phospholipase A_2, hyaluronidase, venom allergen 3, and venom dipeptidyl peptidase IV), and non-classical (e.g. peptidyl-prolyl cis-trans isomerase, phosphoglycerate mutase, and arginine kinase) Hymenoptera venom proteins were identified in *M. pilosula* venom (see Chapter 5 for details of this research). Most of the newly identified IgE-binding proteins were enzymes, two of which, arginine kinase and dipeptidyl peptidase IV, were glycosylated. My analysis identified a positive correlation between recognition to Myr p 1 and a risk of developing adverse reaction to immunotherapy, and a negative correlation between IgE-binding recognition to high molecular weight venom proteins and low-titre reading of venom-specific IgE by ImmunoCAP. This study has for the first time established the complete identity of IgE-binding components in *M. pilosula* venom and their clinical relevance.

Adjuvants are commonly used for their antigen-sparing effect in vaccines and an increasing number of Allergen Immunotherapy products formulated with an adjuvant system are clinically available (306, 310). There is an enormous potential for a safer, more effective Venom Immunotherapy product that utilizes less of the valuable venom extracts compared to current products. Previous attempts to formulate *M. pilosula* Venom Immunotherapy with the adjuvant L-tyrosine revealed the difficulty of incorporating allergenic materials and maintaining their stability in an adjuvant system (350). In the current study, the incorporation of delta inulin (*Advax*™) adjuvant into a low-dose *M. pilosula* Venom Immunotherapy formulation only marginally affected the physicochemical and microbiological stability of the allergen product components for at least 2 days at 4 and 25^{o}C. Importantly, similar results were obtained in products formulated without the adjuvant. *In vivo* studies in mice showed that low-dose *M. pilosula* Venom Immunotherapy formulated with *Advax*™ adjuvant significantly increased venom-specific IgG, consistent with an antigen-sparing effect of the adjuvant (see Chapter 6 for details of this research).

These encouraging results observed in this preclinical study provide supportive data to clinically test *Advax*™ adjuvant in combination with low-dose *M. pilosula* Venom Immunotherapy (Clinical Trial Notification ID: CT-2015-CTN-03308-1 v2; Human Research Ethics Committee approvals: H0015893 and HREC/15/RAH/438, for the Royal Hobart Hospital and Royal Adelaide Hospital, respectively).

7.2. Conclusion

My PhD research has advanced our current understanding on the manufacture, storage and shelf-life of Allergen Immunotherapy products derived from purified *M. pilosula* venom extracts, and the complete identity and clinical relevance of immunologically active components in the venom. Importantly, the results from this study have provided us with the robust scientific evidence to optimize this life-saving treatment in the future.

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